Universidade de Aveiro Departamento de Biologia
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CLARA HOWCROFT FERREIRA

ABORDAGENS MOLECULARES E SUB-CELULARES EM ECOTOXICOLOGIA TERRESTRE

MOLECULAR AND SUB-CELLULAR APPROACHES IN SOIL ECOTOXICOLOGY

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação do Professor Dr. Amadeu Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro, e co-orientação da Dra. Mónica Amorim, Investigadora Auxiliar do CESAM, Departamento de Biologia da Universidade de Aveiro e do Dr. Carlos Gravato, Investigador Auxiliar do CIIMAR, Universidade do Porto.

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palavras-chave

Enquitraídeos, biomarcadores, microarrays, cobre, fenmedifam, propriedades do solo.

resumo

O organismo teste *Enchytraeus albidus* é frequentemente utilizado na avaliação de risco ambiental em solos. Existem testes biológicos padronizados para este organismo (ISO 16387, 2003; OECD 202, 2004) para a determinação de parâmetros tradicionais como a sobrevivência e reprodução (6 semanas). Mais recentemente, têm sido realizados testes de "evitamento" (48h), utilizando a capacidade destes organismos evitarem solos contaminados. Embora se conheçam os efeitos de vários tipos de stress nos níveis crónico e agudo, sabe-se muito pouco sobre os efeitos ao nível sub-celular e molecular. O principal objectivo deste trabalho experimental consistiu na determinação de marcadores de stress oxidativo, neuro-musculares (cholinesterases) e de expressão génica em *E. albidus* expostos a diferentes factores abióticos e aos químicos cobre e fenmedifam durante diferentes períodos de exposição e correlacioná-los com efeitos populacionais e comportamentais. A escolha dos químicos cobre e fenmedifam deveu-se ao facto de representarem dois grupos de stressores químicos com diferentes propriedades, presentes frequentemente nos solos, metais e herbicidas, e devido ao conhecimento prévio dos seus efeitos a nível populacional. A escolha dos parâmetros moleculares e sub-celulares utilizados neste estudo deveu-se às seguintes razões: os biomarcadores de stress oxidativo determinados consistem em defesas antioxidantes que destoxificam espécies reactivas de oxigénio e produtos pró-oxidantes, assim como indicadores de dano oxidativo; as colinesterases são biomarcadores enzimáticos relacionados com funções neuronais e musculares e, em particular, as acetilcolinesterases desempenham um papel central no mecanismo de neurotransmissão; a análise de microarrays permite medir directamente as alterações na regulação da transcrição de centenas de genes simultaneamente. Os resultados obtidos mostram que os biomarcadores de stress oxidativo e neuro-musculares determinados são significativamente alterados em *E. albidus* exposto a diferentes factores abióticos e aos químicos, nos diferentes tempos de exposição. As hibridações dos microarrays revelaram genes diferentemente expressos nas condições testadas. Os resultados obtidos para todos os parâmetros determinados mostram claramente que os solos naturais são mais indicados para testes com *E. albidus*. Os resultados sugerem que as alterações bioquímicas e moleculares observadas em todas as condições podem estar associadas aos efeitos observados em termos de sobrevivência, reprodução e evitamento. Esta tese demonstra a importância da utilização de diferentes ferramentas em ecotoxicologia terrestre e aponta para o uso de novos parâmetros para uma determinação de stress em ecotoxicologia de solos mais rápida e eficaz.

keywords Enchytraeids, biomarkers, microarrays, copper, phenmedipham, soil properties.

abstract

Traditionally, in soil Environmental Risk Assessment (ERA), a set of standardized biological tests are available and commonly performed with *Enchytraeus albidus* (ISO No 16387, 2003; OECD 202, 2004) for the determination of survival and reproduction endpoints (6 weeks test). More recently this organisms' ability to avoid contaminated soil has been used to determine avoidance behaviour (48h test). Though stress effects are known at acute and chronic levels for a wide range of stress conditions, very little is known about the sub-cellular and molecular effects in these organisms. The main objective of this work was to assess oxidative stress, neuro-muscular (cholinesterases) and gene expression markers in *E. albidus* exposed to different soil properties and the chemicals copper and phenmedipham during different exposure periods and to link them with population and behaviour level effects. Copper and phenmedipham were chosen for this study because they are representative of two different groups of chemical stressors with different properties, present commonly in soils, metals and herbicides and due to previous knowledge of effects at population level. The endpoints assessed were chosen for the following reasons: the oxidative stress biomarkers determined consist of antioxidant defences that detoxify reactive oxygen species and prooxidant products, as well as indicators of oxidative stress damage; cholinesterases are biomarker enzymes related with neural and muscular functions and acetylcholinesterase, in particular, plays a central role in the mechanism of neurotransmission; the microarray analysis allows the direct measurement of changes in the transcriptional regulation of many thousands of genes simultaneously. The results showed that the oxidative stress and neuro-muscular biomarkers determined were significantly affected in *E. albidus* exposed to different soil properties and to the chemicals at the different exposure periods. The microarray hybridizations revealed differentially expressed genes for these stress conditions. The results obtained for all endpoints clearly show that natural soils are more appropriate for ecotoxicological testing with *E. albidus*. The results suggest that these biochemical and molecular alterations induced by different soil properties and chemicals might be associated with the effects observed on *E. albidus* survival, reproduction and avoidance behaviour. This thesis shows the importance of using different tools in soil ecotoxicology and points to the use of new endpoints for a quicker stress assessment in soil ecotoxicology.

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Chapter 1

General Introduction

General Introduction

1. Soil ecotoxicology – an introduction

The term "ecotoxicology" was coined by Truhaut in 1969 and is defined as the branch of toxicology concerned with the study of toxic effects of chemicals and physical agents, be they anthropogenic in origin or otherwise, on populations and communities within defined ecosystems, and includes the transfer pathways of those agents and their interactions with the environment (Truhaut, 1977; Butler, 1978). Ecotoxicology is a very broad discipline incorporating aspects of classical and behavioral ecology, toxicology, physiology, molecular biology, environmental chemistry and various other disciplines. The task of ecotoxicology is to assess, monitor and predict the fate and effects of foreign substances in the environment (Moriarty, 1988), and providing scientific knowledge as a basis for evaluations to decide which substances can be released into the environment, or are environmentally tolerable, and threshold values (Römbke & Moltmann, 1996). Ecotoxicology is also the basis for Ecological or Environmental Risk Assessment (ERA), the procedure by which the likely or actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies (Depledge & Fossi, 1994).

Worldwide, many soil systems have been contaminated with chemicals and require some form of mitigation and remediation. However, soil systems are essential for sustaining agricultural practices, perpetuating natural resource industries and maintaining the proper functioning of natural ecosystems. To maintain soil quality, protect human health and the environment from past and current contamination by chemicals, it is imperative that we understand the effects of chemicals on soil organisms and develop a sound regulatory strategy for the protection of soils (Lanno *et al.*, 2003).

Soils are heterogeneous mixtures of a large variety of organic, organo-mineral, and mineral components, as well as soluble substances. The bedrock, the regional climate conditions and the influence of biological factors determine whether and what types of soils evolve (Römbke & Moltmann, 1996). Consequently, there is an

extremely wide variety of soil types with significant differences in composition and characteristics, which are constantly changing, even without human influence (ECETOC, 1990). Therefore the quality and quantity of contaminant binding is highly variable. The manner in which contaminants interact with soil proprieties and organisms is central to understanding their fate, transport, bioavailability and, finally, the occurrence of effects.

The relation between the mobile and immobile contaminant pools is a function of the physico-chemical characteristics of the substance and the composition of the soils (especially contents of organic matter, Fe-, Al- and Mn- oxides, as well as clay), the soil pH and redox conditions. The type of soil substrate selected for a test may have a profound influence on the test organism's response to chemicals, both due to physico-chemical relationships between soil and chemical and the organism's health within the test system (Løkke *et al.*, 2002) and therefore the choice of appropriate substrates for toxicity testing is a major concern. (McLaughlin *et al.*, 2002)

There is a large amount of different soil types, which have different properties causing different interactions with the chemical and organisms. In this study, two different standard soils were used in the bioassays: OECD (Organization for Economic and Cooperation and Development) artificial soil (OECD, 1984) and natural LUFA (Landwirtschaftliche Forschungsanstalt) 2.2 soil. OECD artificial soil has a general acceptance in ecotoxicological testing and therefore is commonly used, due to the advantages in terms of standardization and comparability. It consists of a mixture of sand (70%), kaolinite clay (20%), grounded peat (10%) and calcium carbonate (0.3-1%) for pH adjustment (6.0 ± 0.5). Being artificially made, manipulation of the different constituents is possible, and therefore it allows testing the influence of individual changes in soil properties on organisms. It has however several disadvantages, such as the fact that it is difficult to extrapolate results obtained with artificial soil to field situations, where natural soils differ in terms of texture, structure and chemical characteristics (Løkke *et al.*, 2002; Römbke & Amorim, 2004). Hence, the growing number of critics supports the idea that a natural soil should be used as the standard test substrate (e.g. Van Gestel & Weeks, 2004; Römbke & Amorim, 2004), like LUFA soils. Nevertheless, these

LUFA soils are also a limited solution, as their variance in terms of soil properties is not representative of a broad scale.

2. Test organism

The appropriate selection of the test species is crucial for an adequate risk assessment. Test organisms are frequently used to access the effect of toxic substances and they have been chosen due to several reasons, among which are included the following pre-requisites: easy to handle, short life-cycle, easy to culture and reproduce in laboratory. Species diversity of soil organisms is very high but risk assessment of the effects of chemicals in soil biota has to be restricted to only a few of these organisms due to feasibility reasons.

The test organisms selected for this research project were enchytraeids, more specifically *Enchytraeus albidus* (Figure 3).

Figure 3: *Enchytraeus albidus*.

The family Enchytraeidae belongs to the Phylum Annelida, class Clitellata, order Oligochaeta. Among the microdrile annelid worms, they are the closest relatives to the earthworms (Erséus & Källersjö 2004). They are typical inhabitants of many soils present worldwide, in different abundances. Enchytraeids belong to the saprophagous mesofauna of the litter layer and the upper mineral soil and contribute to vital processes of this environmental compartment. Indirectly they are involved in regulating the degradation of organic matter, as well as improving the pore structure of the soil (Amorim *et al.*, 2005).

Despite their important role in many ecosystems, the Enchytraeidae had been virtually neglected as test organisms until about 15 years ago (Römbke, 2003).

Upon discovery of their sensitivity to anthropogenic stressors in field studies they began to be used frequently in terrestrial ecotoxicological studies and were subjected to various standardization procedures (ISO 2003, OECD 2003 and ASTM, 2004).

Enchytraeus albidus is the best-known and one of the largest species of the genus *Enchytraeus*. It has an average size of about 20 mm. Worldwide it occurs in places where a large amount of organic material is present. *E. albidus* reproduces sexually (Westheide & Müller, 1996) but self-fertilization and parthenogenesis do exist (Gavislov, 1935). *E. albidus* has been used in single chemical assays with OECD artificial soil or LUFA 2.2 natural standard soil, and has also been used successfully for the assessment of soil quality (Jänsch *et al.*, 2005).

3. Traditional endpoints

The effects of chemicals in the environment can only be evaluated if their destiny, metabolism and interactions with the ecological compartment to be protected are known. The best way to obtain a proper perception of these complex processes is through long time studies in the respective ecosystems (Gawlik *et al.*, 1996). However, due to practical constraints, the risk assessment of chemical is done, in first instance through laboratory tests.

Laboratory ecotoxicity tests were developed to evaluate and predict effects in the environment. These effects can be of short duration such as survival (accessed by acute tests) and avoidance behaviour, or long term (in small organisms most of the individual life-cycle, in larger animals, up to one-third of the mature life-cycle) in the case of sublethal parameters (e.g. reproduction, growth), assessed by chronic tests. Moreover, these two kinds of effects also differ in terms of their orientation: acute tests are usually employed as a 'screening tool' with higher toxic concentrations over a broad range, whereas chronic procedures tend to be applied for in-depth investigations, i.e. concentrations are used which do not have any acute effects, or only a low mortality, in the preceding toxicity test (Hamburger, 1983). Some substances have an immediate, lethal effect, whereas others may have impacts which do not manifest until later, e.g. when a population declines as a result of reduced fertility (Römbke & Moltmann, 1996). The kind of effect that is

measured is of different importance and risk assessment criteria must be adjusted accordingly.

Soil ecotoxicology is a relatively young science and therefore a lack of standardised laboratory toxicity tests with soil organisms existed for a long time (Van Gestel, 1998). However, due to the necessity of specific test methodologies so that tests can be reproducible and compared between laboratories and research centres, the situation has improved greatly. At present, acute and chronic toxicity tests with earthworms, collembolans and enchytraeids are standardised by OECD (for single chemicals) and ISO (for soil quality assessment), for the evaluation of effects induced by heavy metals, organic chemicals and, in particular, pesticides.

4. Molecular endpoints

Traditionally, within the framework of the risk assessment of chemicals, the tests performed on soil invertebrate test species are long and/or allow to assess their effect at the population level, on mortality, reproduction or avoidance of contaminated soil. However, there is a need to develop faster, reliable, relevant and ecologically relevant tests to assess the possible risk of chemicals for soil organisms, allowing a holistic view of the biochemical pathways affected. Therefore, recent developments in terrestrial invertebrate ecotoxicology involve the use of biomarkers and genomics, with the goal of using these tools to determine soil contamination, differentiate between different physical and chemical stressors and determine potential environmental hazards and risks on the long term, by focusing on relevant molecular events that occur after exposure.

Risk assessment based only on environmental levels is complicated due to the fact that various pollutants and their derivatives can mutually affect their toxic actions (Calabrese, 1991). Deleterious effects on populations are often difficult to detect in feral organisms since many of these effects tend to manifest only after longer periods of time. When the effect finally becomes clear, the destructive process may have gone beyond the point where it can be reversed by remedial actions or risk reduction. The sequential order of responses to pollutant stress within a biological system is visualized in Figure 4.

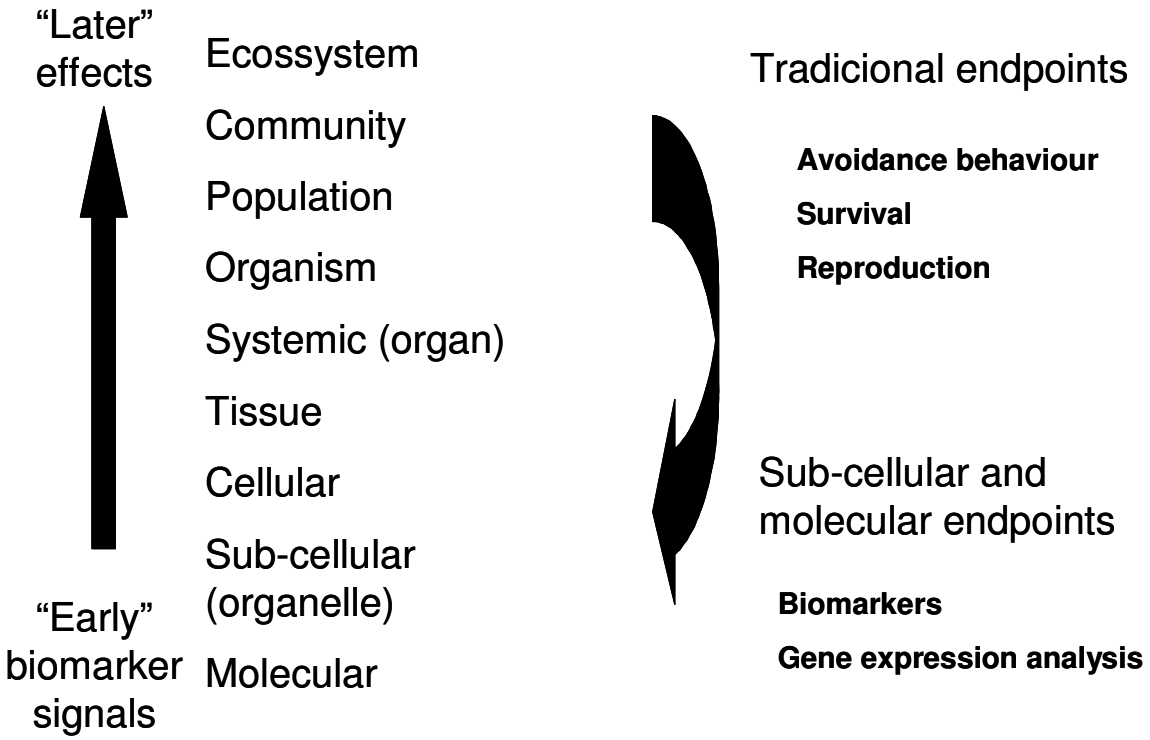

Figure 4: Schematic representation of the sequential order of responses to pollutant stress within a biological system (adapted from Bayne *et al.*, 1985).

Such scenarios have triggered the research to establish early-warning signals reflecting the adverse biological responses towards anthropogenic environmental toxins (Bucheli & Fent, 1995). Effects at higher hierarchical levels are always preceded by earlier changes in biological processes, allowing the development of early-warning biomarker signals of effects at later response levels (Bayne *et al.*, 1985). In an environmental context, biomarkers seem to be promising tools when used as sensitive indicators demonstrating that toxicants have entered organisms, have been distributed between tissues, and are eliciting a toxic effect at critical targets (McCarthy & Shugart, 1990). The development and application of biomarkers in ecotoxicology, has resulted partly due to a desire for early warning indicators that respond before measurable effects on individuals and populations occur, and partly to aid in the identification of the causes of observed population and community-level effects. Whereas older biomarkers focused on measurements of organism physiology or biochemistry, advances in molecular biology are extending the biomarker philosophy to the level of the genes (i.e.,

ecotoxicogenomics). However, although the use of biomarkers seems a promising tool, it is still necessary to determine which biomarkers are in fact useful for discriminating different stresses and are relevant for risk assessment in different organisms and ecological compartments, providing the link between 'lower level effects' and population effects.

5. Objectives/Aims

The main aim of this research project was to determine the effects of copper, phenmedipham and abiotic factors on *E. albidus* at lower levels of organization and relate these endpoints with standard traditional population level and behaviour endpoints. The specific objectives were:

- To study the effects of copper, phenmedipham and abiotic factors on oxidative stress biomarkers in *E. albidus* – **Chapter 2**;
- To characterize the Cholinesterase (ChE) activity in *E. albidus* – **Chapter 3**;
- To determine the *in vivo* and *in vitro* effects of copper, phenmedipham and abiotic factors on ChE activity – **Chapter 3**;
- To study the effects of copper, phenmedipham and abiotic factors on gene expression in *E. albidus* – **Chapter 4**.

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Chapter 2

Effects of natural and chemical stressors on *Enchytraeus albidus*: Can oxidative stress parameters be used as fast screening tools for the assessment of different stress impacts in soils?

Effects of natural and chemical stressors on *Enchytraeus albidus*: Can oxidative stress parameters be used as fast screening tools for the assessment of different stress impacts in soils?

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Abstract

Enchytraeids are important organisms of the soil biocenosis. They improve the soil pore structure and the degradation of organic matter. These organisms are used in standardized testing, using survival and reproduction (6 weeks) as endpoints. The use of biomarkers, linked to ecologically relevant alterations at higher levels of biological organization, is a promising tool for Environmental Risk Assessment. Here, enchytraeids were exposed for different time periods (two days and three weeks) to different soils (OECD artificial soil, different compositions in its organic matter, clay or pH value, and LUFA 2.2 natural soil) and different chemicals (Phenmedipham and copper). The main question addressed in the present study was if the effects of chemicals and different soil properties are preceded by alterations at the sub-cellular level, and if these endpoints may be used reliantly as faster screening tools for the assessment of different stress conditions in soils. The parameters measured in *E. albidus* whole body were: lipid peroxidation (LPO), total glutathione (TG), as well as the enzymatic activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione S-transferase (GST). The results showed that biomarker responses in E. albidus were significantly affected by the soil type (GST, CAT, GPx, GR and LPO) and the duration of exposure in OECD artificial soil (GST, GPx, GR, CAT and LPO) but not in LUFA 2.2 natural soil. For the abiotic factors studied, after 2 days, low pH decreased significantly the TG levels and the activities of CAT and GR,and low OM also significantly decreased CAT and GR activities. After 3 weeks, differences in soil properties caused a decrease in GR and GPx activities, whereas increased GST activity was observed due to low organic matter and pH. Copper significantly increased the activities of CAT, GPx and GR, and decreased the activity of GST after 2 days as well as inscreasing LPO levels after 3 weeks. Phenmedipham increased LPO levels, associated with increased levels of TG as well as increased activities of CAT and GPx and decreased GST activity after 3 weeks exposure. This study shows that both abiotic and chemical stresses could be followed through biomarker analysis and that some of these determinations are potential endpoints in a quick soil contamination assessment procedure.

Keywords: Enchytraeids; Phenmedipham; Copper; Abiotic Factors; Enzymatic and Non-Enzymatic Antioxidants; Oxidative Stress

1. Introduction

Enchytraeids are part of the saprophagous mesofauna of the litter layer and the upper mineral soil, and contribute to vital processes of this environmental compartment. These terrestrial organisms directly improve the pore structure of the soil and are indirectly involved in the regulation of the organic matter degradation (Amorim *et al*., 2005 a). They have been used in ecotoxicology and Environmental Risk Assessment (ERA) to assess the effects of single chemicals or to evaluate soil quality (ISO, 2003; Jänsch et al., 2005). The biological endpoints often used, survival and reproduction, are consistent and important to predict threshold values for policy makers and to screen polluted soils for toxicity. However, these methods are time consuming and may underestimate effects occurring at the molecular and sub-cellular levels which may have impacts later in time, if they are important enough to decrease vital processes (e.g. survival, growth, reproduction, defences against chemical insults) in a sufficient number of individuals.

Over the past few years, increasing emphasis has been placed on the use of biomarkers as early-warning tools for monitoring both environmental quality and the fitness of organisms inhabiting contaminated ecosystems (Stegeman et al., 1992).

The interest in biomarkers gave rise to studies on antioxidant defences (Di Giulio et al., 1989; Winston & Di Giulio, 1991) that play a crucial role in cell homeostasis avoiding DNA damage, enzymatic inactivation and peroxidation of cell constituents due to increased reactive oxygen species (ROS) production (Halliwell & Gutteridge, 1999). When xenobiotics cause physiological responses to deviate beyond typical ranges, then individual fitness may be impaired (Calow, 1991; Calow & Forbes, 1998). Biomarkers that measure toxic effects at the sub-cellular level have been shown to provide rapid quantitative predictions of a toxic effect upon individuals in laboratory studies. Therefore, they should be applied as complementary tools to demonstrate possible links between sub-lethal biochemical alterations and ecologically relevant effects in natural populations inhabiting contaminated ecosystems. To correlate the alterations on individuals to adverse effects at higher levels of biological organization, the biomarker response

should be associated with the impairment of growth, reproductive output or metabolic function that directly affects the organism (Depledge & Fossi, 1994).

Generally, biomarker responses are considered to be intermediates between pollutant sources and higher-level effects (Suter, 1990). When these compensatory responses are activated, the survival potential of the organism may already have begun to decline because the ability of the organism to mount compensatory responses to new environmental challenges may have been compromised (Depledge & Fossi, 1994). In the normal healthy cell, reactive oxygen species (ROS) and prooxidant products are detoxified by antioxidant defences that comprise water and lipid soluble low molecular weight free radical scavengers (like GSH) and specific antioxidant enzymes (Halliwell & Gutteridge, 1999). The balance between prooxidant endogenous and exogenous factors and antioxidant defences in biological systems can be used to assess toxic effects under stressful environmental conditions. Marked increases in ROS production (either directly, or indirectly) can overcome antioxidant defences, resulting in increased oxidative damage to macromolecules and alterations in critical cellular processes, which is designated oxidative stress. Oxidant-mediated effects with a potential suitability as biomarkers include either adaptive responses, such as increased activities of antioxidant enzymes and concentrations of non-enzymatic compounds, or manifestations of oxidant-mediated toxicity such as oxidations of proteins, lipids and nucleic acids, as well as perturbed tissue redox status (Winston & Di Giulio, 1991; Filho, 1996). The response to an oxidative stress challenge is depicted in figure 1.

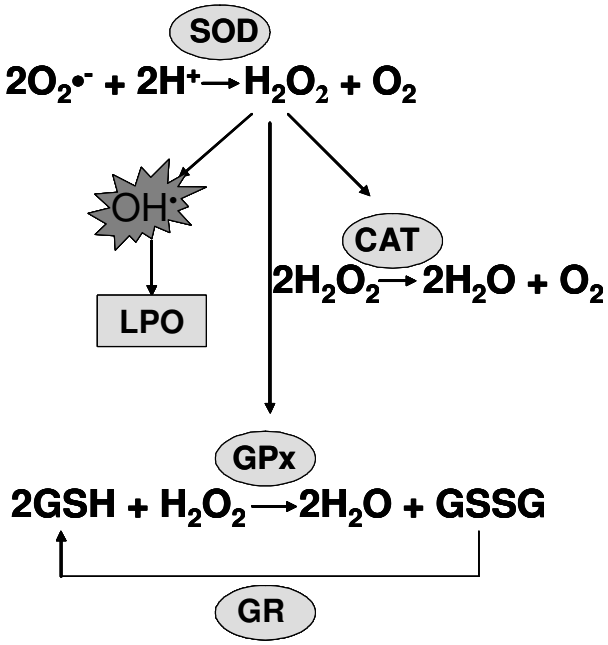


Figure 1: Response to an oxidative stress challenge: the superoxide anion ($O_2\bullet^-$) is metabolised by SOD into molecular oxygen and hydrogen peroxide (H_2O_2), which is then deactivated by CAT; H_2O_2 and organic hydroperoxides are also metabolised by GPx in the presence of GSH; GSH is then regenerated by GR; GST conjugates electrophilic compounds with reduced glutathione; H_2O_2 can also give rise to the hydroxyl radical ($OH^\bullet$) that leads to lipid peroxidantion (LPO).

Establishing the linkage between biomarkers and higher level effects, by choosing the appropriate useful biomarker for discriminating different stresses that are relevant for risk assessment is now the goal of this line of research. Recently, some studies have shown that some biomarkers (e.g. acetylcholinesterase, lactate dehydrogenase, glutathione redox status, lipid peroxides) are in fact related with ecological relevant parameters (Castro et al., 2004; Moreira-Santos et al., 2005; Moreira et al., 2006; Stanek et al., 2006).

The choice of an appropriate biomarker or groups of biomarkers for biomonitoring purposes requires knowledge of a variety of factors that influence the biomarker response (Mayer *et al.*, 1992; Peakall & Shugart, 1993). A number of biotic and abiotic factors can influence the extrapolation of individual biomarkers to the field monitoring of contaminant effects at population and community levels (Adams, 1990; Lagadic et al., 1994). Therefore, the main goal of the present research study

was to investigate the effects of different stress conditions (natural versus artificial soils; different soil properties and 2 chemical compounds) on several biomarkers indicative of pro-oxidant/antioxidant status of *E. albidus*. The selection of the two chemicals, copper and Phenmedipham, was due to their different properties and due to previous knowledge of effects at population level (Amorim et al., 2005a,b,c, 2008b). Copper is a redox cycling metal able to produce ROS, present in soils worldwide and phenmedipham is an organic substance and a commonly used herbicide. Known effects of these chemicals on *E. albidus,* include avoidance behaviour, survival and reproduction (copper chloride: LC_{50}>320mg/kg, AC_{50}=132.6mg/kg, EC_{50}=97mg/kg; Phenmedipham: AC_{50}=50.7 mg/kg, LC_{50}=50 mg/kg and EC_{50}=29.4 mg/kg). Additionally, the effect of exposure time on the biomarker response was also investigated, since these responses may have a transient temporal feature while others can persist for weeks or months with a continued exposure of the organisms.

2. Material and Methods

2.1. Test organism

The test species used here was the enchytraeid *Enchytraeus albidus* (Henle, 1837), one of the largest species of the genus *Enchytraeus* (adults reach 15–40 mm). This species was maintained in laboratory cultures, being bred in moist soil (50% OECD soil, 50% natural garden soil), at 18ºC with a photoperiod of 16:8h (light:dark), and fed once a week with finely ground and autoclaved rolled oats (Cimarrom, Portugal). Details of the culturing process are given in Römbke and Möser (2002).

2.2. Test soils

Two standard soils were used: natural LUFA 2.2 soil, from Speyer, Germany (Lokke & Van Gestel, 1998) and OECD artificial soil (OECD, 1984). Different OECD artificial soils were produced, manipulating its composition in terms of percentage of clay, sand, peat content and pH value. The main characteristics of the test soils used are presented in Table 1.

Table 1: Main characteristics of the test soils, showing approximate values for: particle size distribution (sand, clay, silt), organic matter content (OM), pH and Water Holding Capacity (WHC).

Soil Type	Sand (%)	Clay (%)	Silt (%)	OM (%)	pH ($CaCl_2$ 0.01M)	WHC
LUFA 2.2.	79	13	8	2.36	5.6	48
OECD St.	70	20	-	10	6.3	80
OECD-clay	20	70	-	10	6.4	107.5
OECD-pH	70	20	-	10	5	59
OECD-OM	72.5	22.5	-	5	6.8	47.5

2.3. Test chemicals

Phenmedipham, an herbicide, was applied as the formulation Betosyp formerly known as Betanal (STÄHLER AGROCHEMIE, 157 g/L a.i.) to LUFA 2.2 soil in the following nominal concentrations: 10 and 32 mg a.i./Kg soil DW (Dry Weight). Copper chloride (di)hydrated ($CuCl_2 \cdot 2H_2O$; purity, 99%; molecular weight 170.48 g/mol; Merck, Darmstadt, Germany), was added as an aqueous solution to the soil to give a final concentration of 320 mg/kg DW. The contamination of all test substrates was done by mixing the aqueous solution of the test chemical into the pre-moistened LUFA 2.2 soil. After homogenous mixing, sub-samples of soil were introduced into the individual test vessels. In the case of the metal, the soil was allowed to equilibrate three days before the start of the test as recommended by McLaughlin et al. (2002). All concentrations are given as active ingredient (a.i.) per kg soil (dry weight).

Test concentrations were selected based on previous results (Amorim et al. 2005 a, b, c, 2008b) and the criterion was to select a concentration where chronic effect (reproduction, within the EC_{50} range) was observed, in order to link biomarkers with higher level effects.

2.4. Test procedure

Fifteen adult worms with well-developed clitellum were selected and introduced in a glass vessel (covered afterwards with a parafilm layer with a few holes for airing), each containing 25 g moist soil (40–60% of the maximum WHC) plus food

supply (50 mg of finely ground and autoclaved rolled oats, being half of the amount supplied every week). Seven replicates per treatment were used. The test was carried out at 20ºC with a photoperiod of 16h:8h (light/dark). The duration of the tests was two days (in the absence of food) and three weeks (animals were fed). On 3 weeks exposure vessels, the soil moisture content was adjusted once a week by replenishing weight loss with the appropriate amount of deionised water. At the end of each exposure period the organisms in each replicate were weighed and then frozen in liquid nitrogen and stored at -80ºC until further analysis, without depuration.

2.5. Biochemical analysis

Each replicate containing 15 animals was homogenized in 1400 µL K-Phosphate 0.1M buffer, pH 7.4. Part of the tissue homogenate (200 µL) was separated into a microtube with 4 µL butylated hydroxytoluene (BHT) 4% in methanol for endogenous lipid peroxidation (LPO) determination by measuring thiobarbituric acid-reactive substances (TBARS) at 535nm as suggested by Ohkawa (1979) and Bird & Draper (1984). The remaining tissue homogenate (1200 µL) of each sample was centrifuged for 20 minutes at 10000g (4ºC) to isolate the Post-Mitochondrial Supernatant (PMS). The PMS was divided into six microtubes for posterior analysis of biomarkers and protein quantification (all microtubes were stored at -80ºC). Total glutathione (TG) content was determined in 150 µL treated sample (200 µL sample, 10 µL ultra pure H_20 and 200 µL TCA 12% incubated during 1h at 25ºC) at 412nm, using a recycling reaction of reduced glutathione (GSH) with 100 µL 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase (GR) excess (25 µL from stock with 1U/mL) (Tietze, 1969; Baker et al., 1990). Glutathione-*S*-Transferase (GST) activity was determined in 50 µL of PMS with 1850 µL K-Phosphate 0.2 M (pH7.9) following the conjugation of 50 µL GSH (0.0251 g in 10 mL H_2O_2) with 50 µL 1-chloro-2,4-dinitrobenzene (CDNB) (0.0827 g in 20 mL ethanol and 30 mL H_2O_2) at 340nm (Habig *et al*., 1974). Glutathione Peroxidase (GPx) activity was determined in 50 µl PMS with 840 µl K-Phosphate 0.05M (pH 7.0) with EDTA 1 mM, Sodium azide 1 mM and GR (7.5 mL from stock with 1U/mL), and 50 µL GSH 4 mM, by measuring the decrease in NADPH (50 µl,

0.8mM) at 340nm and using H_2O_2 (10 µl, 0.5mM) as substrate (Mohandas et al., 1984). GR activity was assayed in 50 µl of PMS based on the method described in Cribb et al. (1989), by monitoring the decrease of NADPH levels (0.0086g in 50 mL K-Phosphate 0.05 M pH7.0, together with0.0327 g GSSG and 0.0098 g DTPA), using 950 µl of reaction buffer, at 340nm. Superoxide dismutase (SOD) activity determination in 50 µL PMS was based on the reduction of cytochrome c (200 µL of reaction solution (stock: 2mL xanthine 0.7mM and 20mL citocromo C solution 0.03mM) by superoxide radicals produced by the xanthine-xanthine oxidase system (50 µL xanthine oxidase solution (0.3U/ml) and monitored at 550nm (McCord & Fridovich, 1969) adapted to microplate. Catalase (CAT) activity was determined in 50 µl of PMS and 950 µL K-Phosphate 0.05M (pH 7.0) by measuring decomposition of the substrate H_2O_2 (500 µL, 0.030M) at 240nm (Clairborne, 1985). The protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as a standard.

2.6. Statistical analysis

Results were analysed through a two-way ANOVA (SPSS, 1997) for differences between treatments. Holm-Sidak test was used to discriminate statistical significant differences by performing multiple comparisons relatively to the control group. Data was log10 transformed due to lack of normal distribution and homogeneity of variance.,

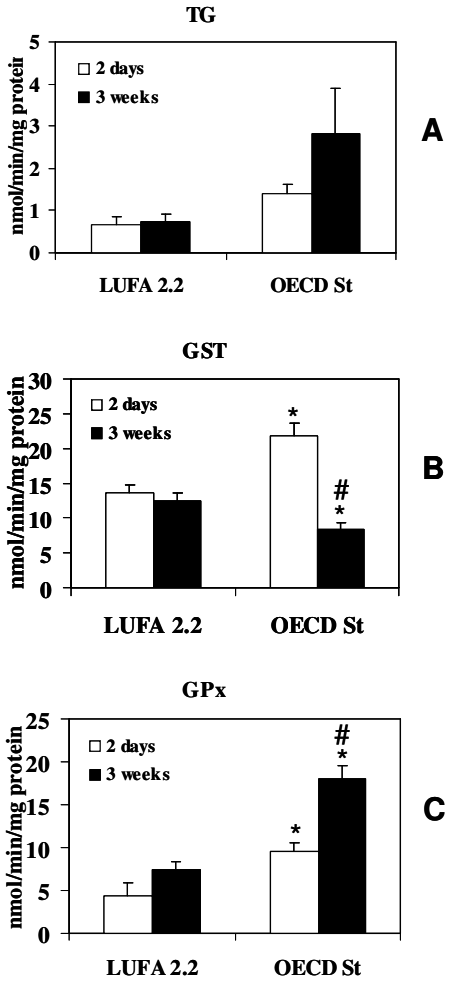
3. Results

LUFA 2.2 natural soil versus OECD artificial soil and the influence of the test duration

The results showed significant differences on biomarkers of *E. albidus* exposed for 2 days or 3 weeks to LUFA 2.2 and OECD soils (Figure 2). The main significant differences after 2 days were higher GST, GPx, GR and CAT ($p<0.05$) in organisms exposed to the artificial soil than in LUFA 2.2 soil (Figure 2B, 2C, 2D and 2F). After 3 weeks, high GPx ($p<0.05$) was still observed in *E. albidus*

exposed to OECD compared to LUFA 2.2 (Figure 2C), as well as high LPO ($p<0.05$) (Figure 2G) and low GST activity ($p<0.05$) (Figure 2B).

The biomarkers measured in *E. albidus* in LUFA 2.2 were not significantly altered with the increasing exposure, whereas biomarkers from organisms exposed to OECD soil showed differences between the two exposure periods. GST, GR and CAT ($p<0.05$) (Figure 2B, 2D and 2F) were significantly decreased at 3 weeks when compared to 2 days, whereas GPx and LPO ($p<0.05$) (Figure 2C and 2G) were significantly increased in organisms exposed to OECD soil.



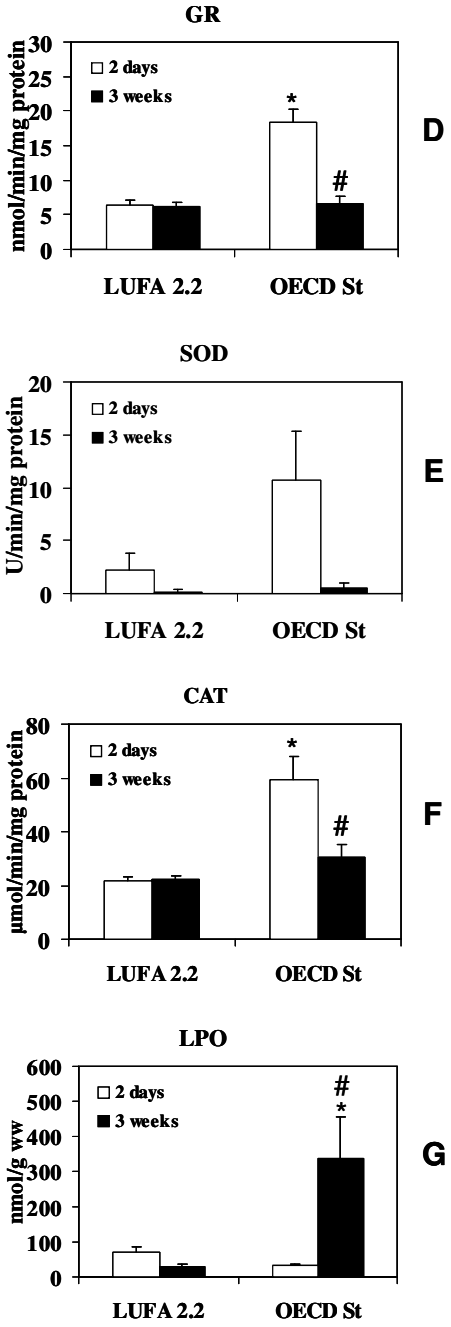
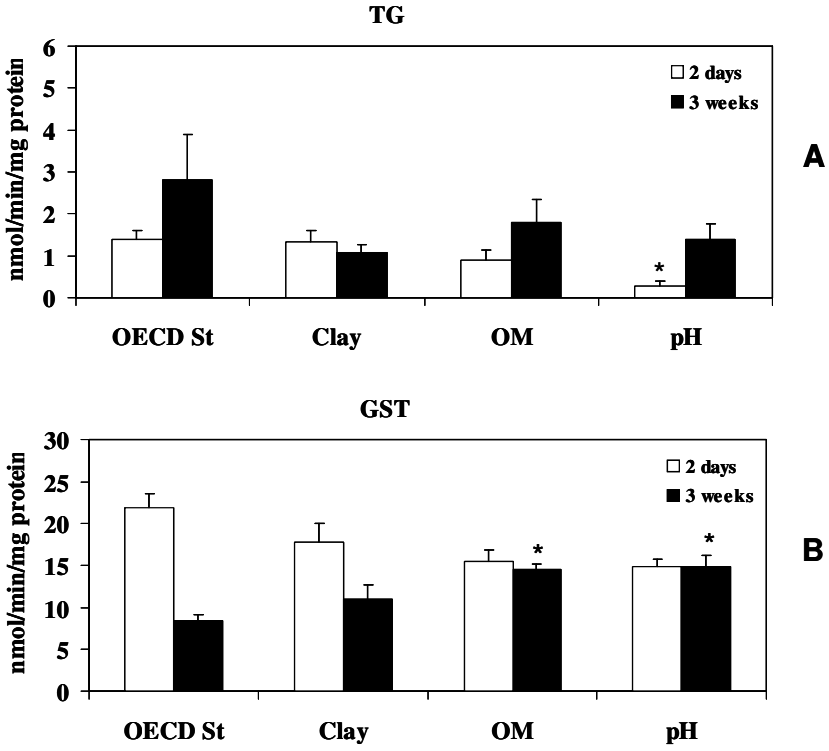
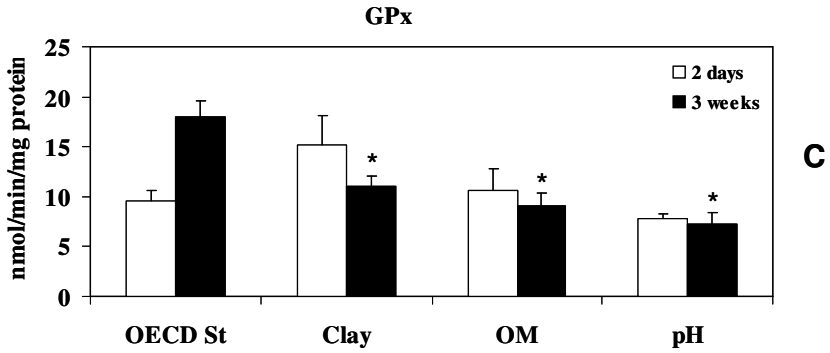


Figure 2: Results of total glutatione (TG), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO) expressed as mean values (± standard error) for *E. albidus* exposed to LUFA 2.2 soil and OECD standard soil (OECD St) for 2 days and 3 weeks. (* indicates statistically significant differences within the same period for the two soils and # indicates statistically significant differences within the same soil for the two periods, Holm-Sydak $p<0.05$).

Effects of different soil properties

The results showed that TG levels as well as GR and CAT ($p<0.05$) activities were significantly lower in *E. albidus* exposed during 2 days to soils with low pH, compared to the control (Figure 3A, 3D and 3F). After 2 days exposure, OECD soil with a low organic matter content decreased *E. albidus* GR and CAT activities ($p<0.05$) (Figure 2D and 2F). Low pH induced a decrease on the LPO levels in comparison with the standard OECD at 3 weeks exposure (Figure 3G). After 3 weeks, low GPx activities ($p<0.05$) were observed in *E. albidus* exposed to soils with high clay content, low organic matter and low pH, compared to the control (Figures 3C). Moreover, high GST activity ($p<0.05$) was observed in organisms exposed for 3 weeks to soils with low organic matter or pH (Figure 3B).




GPx
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15
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2 days
3 weeks
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OECD St
Clay
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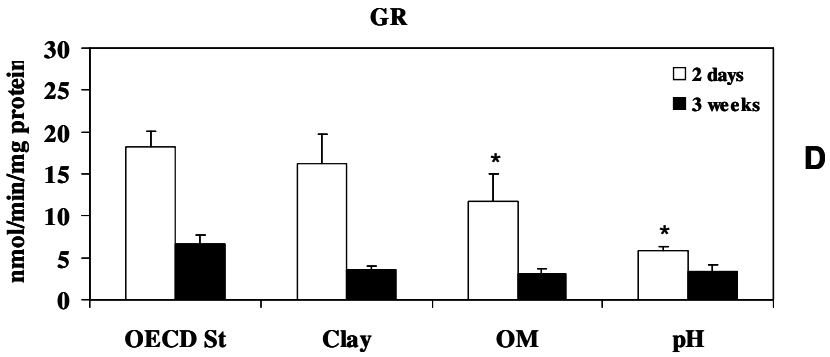
pH

C


GR
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OECD St
Clay
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pH

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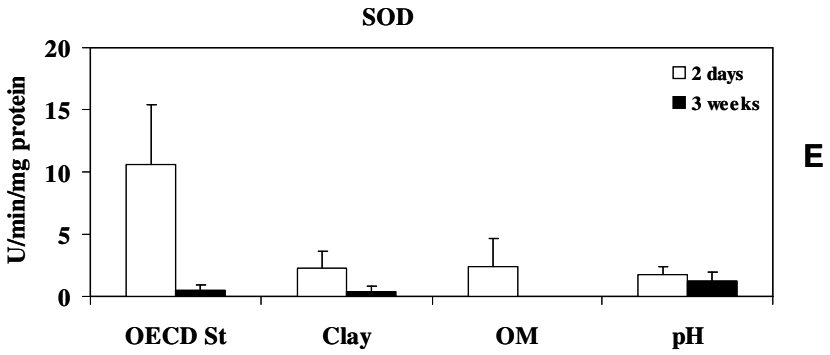
SOD
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OECD St
Clay
OM
pH

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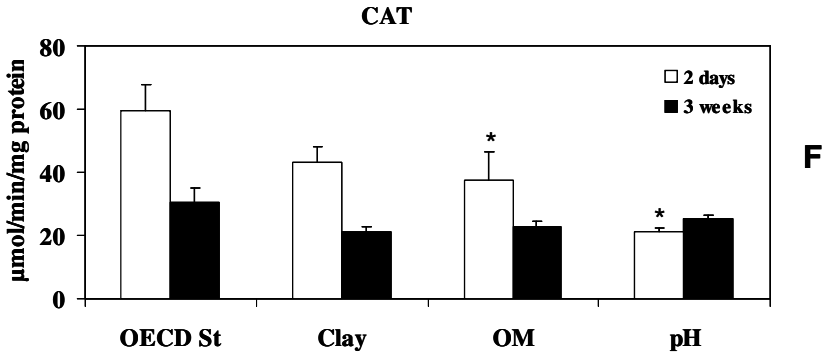
CAT
µmol/min/mg protein
80
60
40
20
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2 days
3 weeks
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OECD St
Clay
OM
pH

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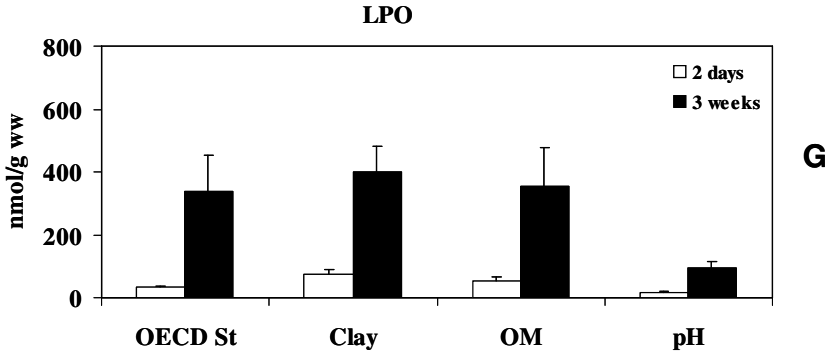
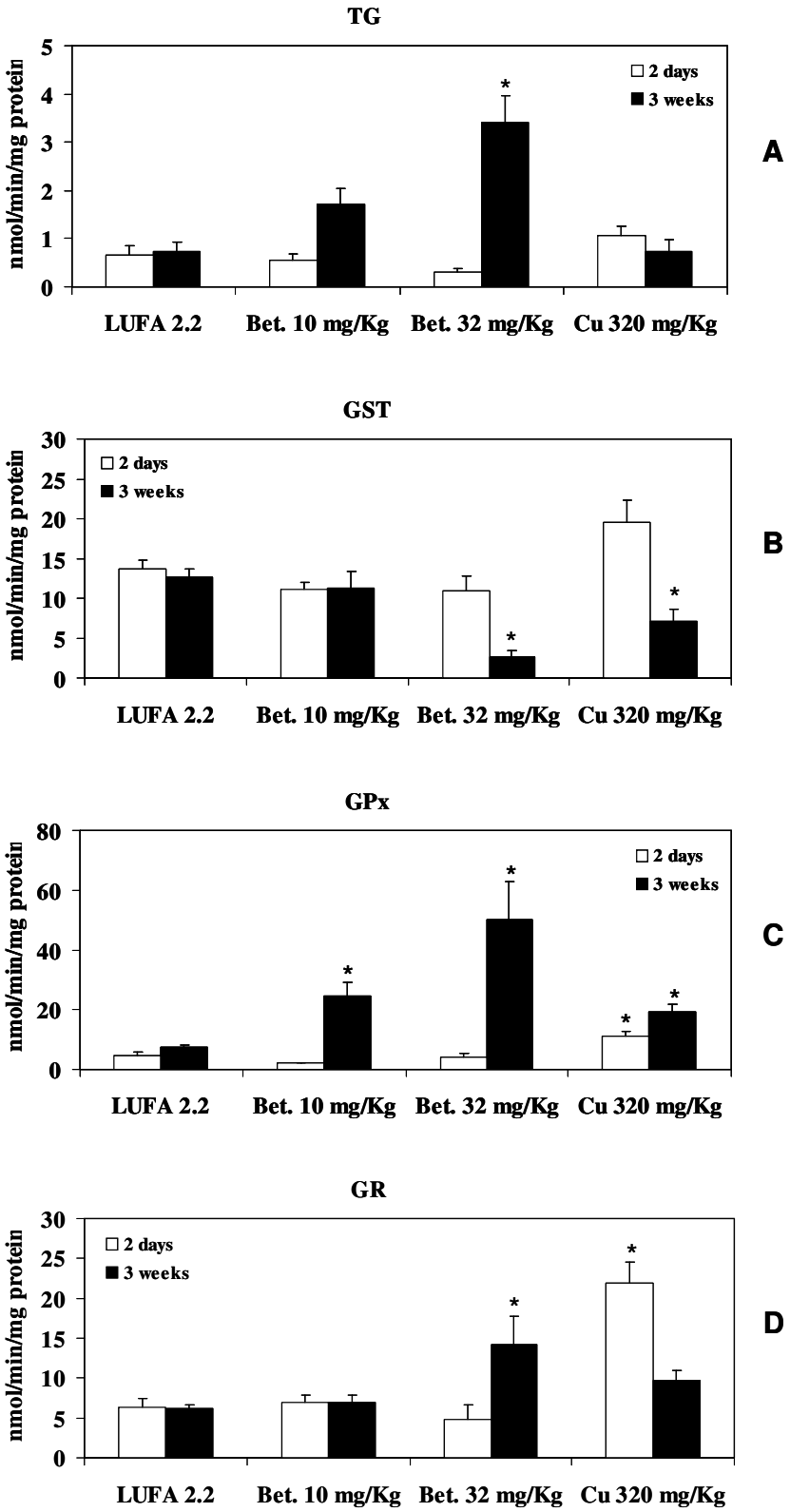


Figure 3: Results of total glutatione (TG), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO) expressed as mean values (± standard error) for *E. albidus* exposed to OECD standard soil (OECD St), OECD soil with a high clay content (Clay), OECD soil with a low organic matter content (OM) and OECD soil with a low pH (pH), for 2 days and 3 weeks. (* indicates statistically significant differences, Holm-Sydak $p<0.05$).

Effect of chemicals

GR, GPx and CAT ($p<0.05$) activities were significantly increased in *E. albidus* when exposed for 2 days to 320 mg/Kg copper, compared to controls (Figure 4B, 4C, 4D and 4F). After 3 weeks, 320 mg/Kg copper induced a significant increase of GPx and CAT activities as well as LPO levels ($p<0.05$) (Figure 4C, 4F and 4G), and a significant decrease of GST activity ($p<0.05$) (Figure 4B), when compared to control. Betanal (10 and 32 mg/Kg) did not significantly alter the biomarker responses evaluated on *E. albidus* exposed during 2 days (Figure 4). However, after 3 weeks exposure to 32 mg/Kg betanal, TG levels and GR activity ($p<0.05$) (Figure 4A and 4D) were significantly increased when compared to control, and GST activity was significantly decreased ($p<0.05$) (Figure 4B). Moreover, both concentrations of betanal induced high GPx and CAT activities ($p<0.05$) (Figure 4C and 4F) as well as LPO levels ($p<0.05$) (Figure 4G) in organisms exposed for 3 weeks when compared to control.


TG
nmol/min/mg protein
2 days
3 weeks
LUFA 2.2
Bet. 10 mg/Kg
Bet. 32 mg/Kg
Cu 320 mg/Kg
A
GST
B
GPx
C
GR
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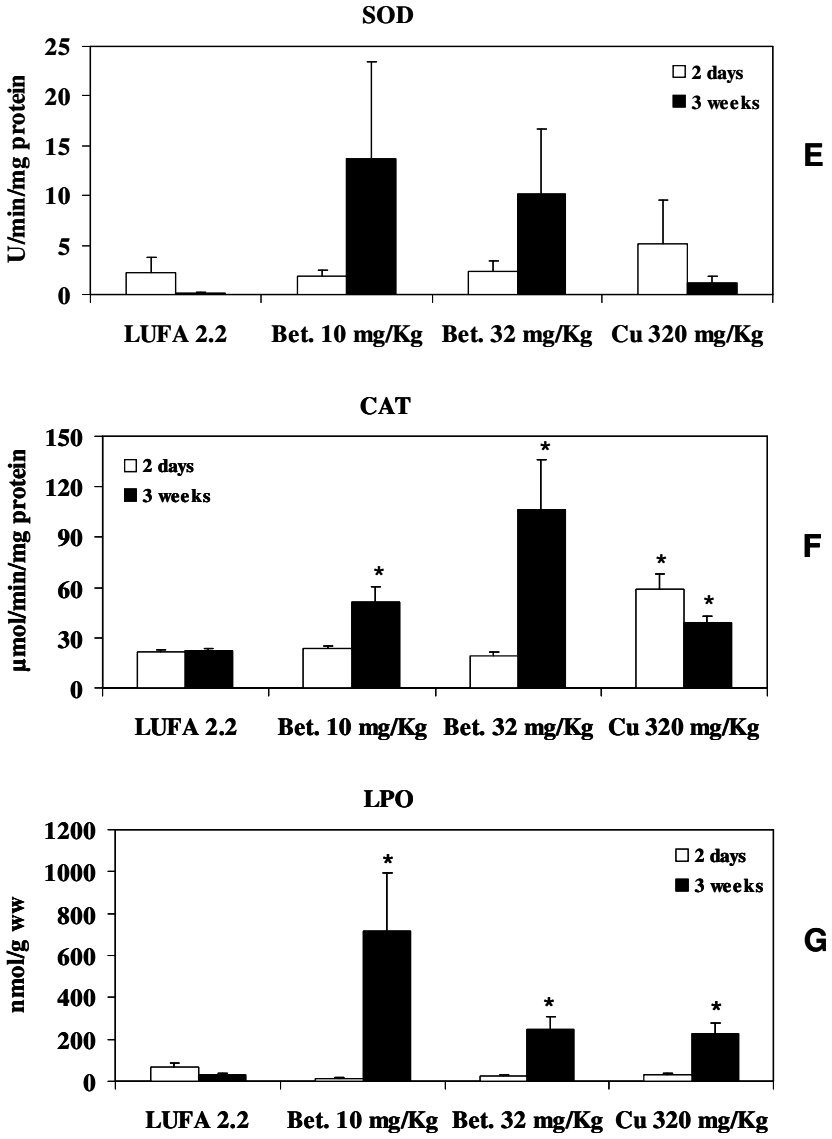

Figure 4: Results of total glutathione (TG), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR) superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO) expressed as mean values (± standard error) for *E. albidus* exposed to LUFA 2.2 control soil and contaminated with Betanal (Bet.) and Copper (Cu) for 2 days and 3 weeks. (* indicates statistically significant differences, Holm-Sydak $p<0.05$).

4. Discussion

Soil type and properties

LUFA 2.2 seems to be the most appropriate soil for *E. albidus,* since the biomarker responses analysed were stable and not time-dependent. In contrast, the results observed when *E. albidus* were exposed to OECD soil were highly variable and time-dependent.

Moreover, high levels of LPO were observed on organisms exposed during 3 weeks to OECD soil, which is a clear indication that they were facing an oxidative stress status, compared to the ones in LUFA 2.2. Previous studies performed with *E. albidus* and *E. crypticus* also showed that the substrates used for culturing these organisms influence oxidative stress biomarkers (Suteková et al., 2007). Moreover, a comparative study with the earthworm *Eisenia fetida* demonstrated that exposure to non-natural substrates increased the levels of GSH as well as the activities of GR and GST (Arnaud et al., 2000). Current results are in good agreement with previous studies using *E. albidus,* where the reproductive output is higher in LUFA 2.2 than in OECD soil (Amorim et al., 2005b) and that OECD soil is avoided when LUFA 2.2 soil is available, during the avoidance tests (Amorim et al., 2005a). Overall, the biomarkers values/activities determined tend to be higher in OECD soil. Therefore, *E. albidus* exposed to OECD soil is facing an oxidative stress problem that might be linked with the avoidance behaviour and decreased reproductive output.

Previous studies concerning avoidance and reproduction tests (Amorim et al. 2008a) clearly showed that *E. albidus* prefer non-modified OECD standard soil than OECD soils with modifications of pH, clay and organic matter content. Current results showed that when *E. albidus* is exposed to manipulated OECD soils the antioxidant defences are decreased and GST increased. Organisms exposed to OECD soil with a low pH (5) exhibited high GST activity and low activities of CAT, GPx, GR as well as TG levels, demonstrating an increment of the conjugation capacity associated with an enzymatic antioxidant decrease. Such result was expected since *E. albidus* is an acidophobe (optimum: 6.8 to 7.0)

(Jänsch et al., 2005) and is considered an indicator of slightly acid to slightly alkaline conditions, never to be found in strongly acid soils (Graefe & Schmelz, 1999). However, phase II conjugation seems to be increased and this may explain the absence of oxidative damage assessed as LPO. Nevertheless, the decreased antioxidant capacity observed in *E. albidus* can be used as an early-warning parameter since previous studies (Amorim et al*.,* 2005a) showed that these organisms survived but were not able to reproduce in natural soils with low pH. More recently, it was shown that the number of juveniles is decreased when *E. albidus* is exposed to OECD soil with low pH (Amorim et al., 2008a). In soils with low organic matter (containing only 5% of organic matter) an impairment of antioxidant defences (increased GST and decreased GPx, GR and CAT) was observed, but it was not associated with LPO. OECD soil with low OM content also induced effects in the avoidance behaviour and decreased reproduction of *E. albidus* (Amorim et al., 2008a). Morever, Dirven-van Breemen et al. (1994), showed that *E. albidus* reproduction decreases slightly if the organic content is as low as 5%. OECD soil with high clay content decreased the TG level, GPx activity and also affected slightly GR and CAT activities. These changes in enzymatic and non-enzymatic antioxidants were however not associated with high LPO. These results are in accordance with the reproduction tests performed by Amorim et al. (2005a) with natural soil types, showing that although *E. albidus* adult survival was not affected by high clay content, these organisms were not able to reproduce. Moreover, reproduction tests performed by Amorim et al. (2008a) with OECD artificial soil showed that, although there were no significant differences in the number of adults between OECD standard soil and OECD soil with high clay content, there were highly significant differences on the number of juveniles. Overall results with manipulated soils showed a clear decrease of the antioxidant capacity of *E. albidus*. According to Stone et al. (2002), enzymatic decreases below normal limits may effectively identify populations that are experiencing stressful conditions. It also seems that the alterations on biomarker responses are associated with effects on *E. albidus* behaviour, reproduction and survival. However, further studies should be performed to better define the preferred range of soil properties for *E. albidus*, and the joint influence of several changing factors.

Chemical contamination

Copper increased the activities of CAT, GR, GPx and GST after 2 days exposure, and after 3 weeks this was still observed for CAT and GPx. These results are consistent with the enzymatic activities in response to an oxidative stress challenge. A previous study, using the earthworm *Aporrectodea tuberculata* exposed up to 7 days to copper showed that GST activity increased with the increase in metal concentration (Lukkari et al., 2004). However, this study also reported that GST increase was transient as our current results show when enchytraeids were exposed to copper for 3 weeks. Studies using *Eisenia fetida/andrei* exposed to lead acetate (Saint-Denis et al., 2001) and *Tubifex tubifex* exposed to Cu during more than 7 days (Molesh et al. 2006) also showed similar short-term time dependence in biomarker responses, including GST. Furthermore, it was shown that GST is involved in the detoxification metabolism of earthworms, controlling and regulating intracellular homeostasis, thereby protecting from damage caused by metal contamination (Dallinger, 1993; Josephy, 1997). For a review on GST modulation in earthworms see Scott-Fordsmand & Weeks (2000).
The enhancement of the antioxidant processes on short exposures to copper (2 days) seems to prevent LPO. However, after 3 weeks exposure to copper it is visible that lipid peroxidation increases significantly due to an antioxidant capacity decrease. These findings are consistent with the known fact that metals, such as copper, and their chelate complexes are implicated in lipid peroxidation and subsequently in the promotion of carcinogenesis (Kasprzak, 1995). Although the results obtained by Amorim et al. (2005a,b,c; 2008b) show that adult mortality LC_{50} for copper is higher than 320 mg/Kg, our results seem consistent with the 97 mg/Kg EC_{50} for reproduction and the 132.6mg/Kg AC_{50} for the avoidance test, showing that these biomarkers are good sublethal stress markers that might be associated with effects at higher levels of biological organization. Moreover, previous research works performed with *E. albidus* exposed to other metals (Cd, Zn and Pb) also showed that alterations on biomarkers (increased LDH and decreased Pyruvate Kinase) were associated with decreased reproductive rates (De Coen et al., 1999).

Betanal seems to have no significant effect on the biomarkers determined on *E. albidus* exposed during 2 days. However, the results after 3 weeks exposure showed high LPO that were associated not only with high GPx and CAT activities for both concentrations tested, but also with high TG and GR, as well as low GST for 32 mg/Kg betanal. These results seem to be in good agreement with decreased reproduction observed on *E. albidus* exposed 3 weeks to betanal (EC_{50}= 29mg/Kg) (Amorim *et al.*, 2005a,b,c; 2008b). Studies performed with other pesticides on earthworms and aquatic worms have also reported sub-cellular and molecular effects, demonstrating their oxidative stress response: e.g. acetocholor has been shown to induce sublethal toxicity response in *Eisenia fetida*, by increasing GST activities in a dose-dependent relation (Xiao, 2006). Isoproturon increased the activity of GST, GR and CAT in a dose-related and time-dependent manner in *Tubifex tubifex* (Mosleh, 2005).

Enzymatic induction above normal ranges may result in reduced fitness because of the energetic demands imposed (Sibly & Calow, 1989). Besides lower maintenance and reproduction (Berenbaum & Zangerl, 1994), additional impacts of enzymes induction may include greater susceptibility to subsequent stressors and reduced performance. The effects on biomarkers in the present study can be compared with previous results at the reproduction level and avoidance behaviour obtained by Amorim et al. (2005a,b,c; 2008b). These biomarkers might be associated with effects at higher levels of biological organization, or even be indicative of them, representing ecologically relevant tools to detect the presence of contaminants in soils.

Further studies should include the determination of oxidative stress biomarkers for other metals, herbicides and soil properties. This will ultimately lead to the establishment of enchytraeids biomarkers which can be used for quick assessment of contaminated land, similarly to what has already been accomplished with earthworms (Hankard et al., 2004; Bundy et al., 2004;; Weeks et al., 2004; Booth et al., 2005), where biomarkers were used to determine metal and organic exposure in contaminated sites.

5. Conclusions

LUFA 2.2 natural soil is more appropriate for *E. albidus* testing than OECD artificial soil. High clay content or low pH and OM influences the levels of enzymatic antioxidants and non-enzymatic antioxidants.

Copper increased the activities of non-enzymatic and enzymatic antioxidants after 2 days exposure. However, oxidative damage was only observed on *E. albidus* exposed for 3 weeks. The effects of betanal on biomarkers of *E. albidus* were observed after 3 weeks exposure, where a concomitant increase on enzymatic antioxidants and oxidative damage was observed for both betanal concentrations.

Test duration influenced the oxidative stress parameters measured. Overall, the test duration that seems more robust for the biomarker analysis is 3 weeks.

This study shows the association of the effects on biomarkers with the alterations on higher levels of biological organization (survival, avoidance and reproduction endpoints), highlighting the ecological relevance of biomarkers, which can be used as early-warning tools in biomonitoring studies.

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Chapter 3

Characterization of Cholinesterases in *Enchytraeus albidus* and assessment of in vivo and in vitro effects of different soil properties, copper and phenmedipham

Characterization of Cholinesterases in *Enchytraeus albidus* and assessment of *in vivo* and *in vitro* effects of different soil properties, copper and phenmedipham

Howcroft, C.F., Gravato, C., Amorim, M.J.B., Guilhermino, L. & Soares, A.M.V.M.

Abstract

Enchytraeus albidus are important organisms of the soil biocenosis, used as tests species for Environmental Risk Assessment. The standardized test procedures have survival and reproduction (6 weeks) as endpoints, as well as avoidance assays (48h). However molecular and sub-cellular studies, as well as biochemical characterizations of this test organism are still scarce. This study aimed to characterize the cholinesterases in *E. albidus* whole body, by using specific substrates and inhibitors. There appears to be only one ChE, which has mixed properties of vertebrate AChE and invertebrate ChE. The effect of different soil proprieties (organic matter, pH and clay content) as well as the effect of Copper and Phenmedipham on Cholinesterases was studied *in vivo*, after 2d and 3 weeks exposure. ChE activities in *E. albidus* were not inhibited by the soil properties exposure, however copper and phenmedipham inhibited ChE activity after 3 week exposure. Additionally, *in vitro* effects of Phenmedipham and Copper were also studied and ChE activity was increasingly inhibited by increasing concentrations/amounts of these two chemicals.

Keywords: Enchytraeids; Cholinesterases; Phenmedipham; Copper; soil proprieties.

1. Introduction

Enchytraeus albidus belongs to the family Enchytraeidae and is part of the saprophagous mesofauna of the litter layer and the upper mineral soil. These organisms contribute to vital processes of this environmental compartment indirectly by regulating the degradation of the organic matter and directly by improving the pore structure of the soil (Amorim *et al*., 2005 a). They have been used in ecotoxicology and Environmental Risk Assessment (ERA) to assess the effects of single chemicals or to evaluate soil quality (ISO, 2003; Jänsch et al., 2005), using survival, reproduction and avoidance behaviour endpoints. Over the past few years, increasing emphasis has been placed on the use of biomarkers as early-warning tools for monitoring both environmental quality and the fitness of organisms inhabiting contaminated ecosystems (Stegeman et al., 1992), and the use of oxidative stress biomarkers and linkage to higher levels effects has recently been addressed (Howcroft et al., 2008).

Cholinesterases (ChE) are interesting biomarker enzymes related with neural and muscular functions (Payne et al., 1996), distributed widely throughout the animal kingdom. There are two broad groups of ChE classified as acetylcholinesterase AChE with a high affinity for acetylcholine, and butyrylcholinesterase (BChE, also called pseudocholinesterase or non-specific cholinesterase), with a high affinity for butyrylcholin (Walker & Thompson, 1991; Massoulié et al., 1993), based on their properties and function in vertebrates. In vertebrates, only AChE is present in the brain, and in muscle tissues both AChE and BChE maybe present (Sturm et al., 2000).

AChE plays a central role in the mechanism of neurotransmission, since it promotes the cleavage of the neurotransmitter acetylcholine after its release at the nervous cleft of cholinergic synapses, preventing overstimulation of the post-synaptic membrane and hence preventing continuous nerve fringing, which is vital for normal functioning of sensory and neuromuscular systems (Nachmansohn & Wilson, 1951). When inhibition of AChE occurs, large amounts of acetylcholine accumulate in the nervous cleft, leading to overstimulation of cholinergic receptors, which is often responsible for acute effects, frequently resulting in death of the exposed organisms. Several classes of environmental contaminants including

pesticides like organophosphate and carbamate, among others (Davies & Cook, 1993; Gill et al., 1990a; Guilhermino et al., 1998; Dembele et al., 1999) and heavy metals (Gill et al., 1990b; Reddy & Venugopal, 1993; Devi & Fingerman, 1995; Labrot et al., 1996; Guilhermino et al., 1998; Frasco et al., 2005) have the potential to decrease AChE in several exposed vertebrates and invertebrates.

Due to their overlapping hydrolytic capabilities, despite the preference of anti-ChEs compounds for AChE, interactions might also occur with other enzymes with cholinesterasic activity present in the same species and tissue. Since the different enzymatic forms may have distinct sensitivities to environmental contaminants (Magnotti et al., 1994), it is crucial to characterise these forms present in species and/or tissues to be used in ecotoxicological studies (Sturm et al., 1999), which is done with the aid of diagnostic substrates and inhibitors (Silver, 1974).

Establishing the linkage between biomarkers and higher level effects is now the goal for biomarker research. Recent studies have proven a possible association between the inhibition of AChE with behaviour alterations in vertebrates and invertebrates.

Effects at the population level, avoidance behaviour and on oxidative stress biomarkers in *E. albidus* due to soil properties including pH, organic matter and clay content, as well as to the chemicals copper and phenmedipham have been previously assessed (Amorim et al., 2005 a, b, c, 2008 a, b; Howcroft et al, 2008). Copper is present in soils worldwide and phenmedipham is an organic substance and a commonly used herbicide. Known effects of these chemicals on *E. albidus,* include avoidance behaviour, survival and reproduction (copper chloride: AC_{50}=132.6mg/kg, LC_{50}>320mg/kg, EC_{50}=97mg/kg; Phenmedipham: AC_{50}=50.7 mg/kg and 7mg/kg, LC_{50}=50 mg/kg and EC_{50}=29.4 mg/kg).

The goals of this study included: **1)** characterization of the ChE forms present in *E. albidus* using specific inhibitors and substrates; **2)** determination of the *in vivo* effects of abiotic factors and copper and phenmedipham during two exposure periods (2days and 3weeks) and **3)** determination of the in vitro effects of copper and phenmedipham.

2. Material and Methods

2.1. Test organism

Laboratory cultures of the test species *Enchytraeus albidus* (Henle, 1837) were maintained in moist soil (50% OECD soil, 50% natural garden soil), at 18ºC with a photoperiod of 16:8h (light:dark), and fed once a week with finely ground and autoclaved rolled oats (Cimarrom, Portugal). Details of the culturing process are given in Römbke and Möser (2002).

2.2. ChE characterization

2.2.1. Test procedure

Two replicates containing 7 organisms with well-developed clitellum, selected directly from the culture, were frozen in liquid nitrogen and stored at -80ºC until further analysis. They were then homogenized in Buffer K-Phosphate 0.1M, pH 7.4. The tissue homogenate of each sample was centrifuged during 20 minutes at 10000 g (4ºC) to isolate the Post-Mitochondrial Supernatant (PMS). The PMS was then used to determine the ChE activity in the presence in different substrates and inhibitors.

The ChE activity was determined, in triplicate, in the PMS, by the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996), using acetylthiocholine as substrate, 50 µl of homogenate and 250 µl of the reaction solution (30ml Buffer K-phosphate 0.1M pH=7.2, 1ml 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) 10 mM with $NaHCO_3$ 17.855 mM in K-Phosphate buffer and 0.2ml acetylthiocholine 0.075M solution). The absorbance was measured at 412 nm during 15 minutes.

The protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as a standard. After testing a range of protein concentrations, the protein content was adjusted to 0.7mg/ml.

2.2.2. Substrates

E. albidus ChE substrate preferences were investigated by determining the enzyme activity, in four replicates, at 12 increasing concentrations, from 0.012 to 24.576 mM, of the substrates acetylthiocholine iodide, s-butyrylthiocholine iodide and propionylthiocholine iodide. ChE activity in the presence of these substrates was determined as described above, with the substrates being added directly to the reaction buffer.

2.2.3. Inhibitors

Eserine sulphate, iso-OMPA (tetraisopropyl pyrophosphoramide) and BW284C51 (1,5-bis-(4-allyldimethyl-ammoniumphenyl)-pentan-3-one dibromide) were used as specific inhibitors of ChE, butyrylcholinesterase (BChE) and AChE, respectively. The inhibitors were added directly to the reaction buffer and ChE activities were measured, in four replicates, at 9 increasing concentrations from 0.781 to 800 µM, as described above.

2.3. *In vivo* effects of soil proprieties, copper and betanal on ChE activity

2.3.1. Test soils

Two standard soils were used: natural LUFA 2.2 soil, from Speyer, Germany (Lokke & Van Gestel, 1998) and OECD artificial soil (OECD, 1984). Different OECD artificial soils were produced, manipulating its composition in terms of percentage of clay, sand, peat content and pH value. The main characteristics of the test soils used are presented in table 1.

Table 1: Main characteristics of the test soils, showing approximate values for: grain size distribution (sand, clay, silt), organic matter content (OM), pH and Water Holding Capacity (WHC).

Soil Type	Sand (%)	Clay (%)	Silt (%)	OM (%)	pH ($CaCl_2$ 0.01M)	WHC
LUFA 2.2.	79	13	8	2.36	5.6	48
OECD St.	70	20	-	10	6.3	80
OECD-clay	20	70	-	10	6.4	107.5
OECD-pH	70	20	-	10	5	59
OECD-OM	72.5	22.5	-	5	6.8	47.5

2.3.2. Test chemicals

Phenmedipham, an herbicide, was applied as the formulation Betosyp formerly known as Betanal (STÄHLER AGROCHEMIE, 157 g/L a.i.) to LUFA 2.2 soil in the following nominal concentrations: 10 and 32 mg a.i./Kg DW (Dry Weight). Copper chloride (di)hydrated ($CuCl_2 \cdot 2H_2O$; purity, 99%; molecular weight 170.48 g/mol; Merck, Darmstadt, Germany), was added as an aqueous solution to the soil in order to give a final concentration of 320 mg/kg DW. The contamination of all test substrates was done by mixing the aqueous solution of the test chemical into the pre-moistened LUFA 2.2 soil. After homogenous mixing, sub-samples of soil were introduced into the individual test vessels. In the case of the metal, the soil was allowed to equilibrate three days before test start as recommended by McLaughlin (2002).

Concentrations were selected based on previous results (Amorim et al. 2005 a, b, c, 2008 a, b), being the selection based on selecting a chronic (reproduction) effect concentration, within the EC_{50} range.

2.3.3. Test procedure

Fifteen adult worms with well-developed clitellum were selected and introduced in a glass vessel (covered afterwards with a parafilm layer in which a few holes for airing were made), each containing 25 g moist soil (40–60% of the maximum WHC) plus food supply (50 mg of finely ground and autoclaved rolled oats, being half of the amount supplied every week). Seven replicates per treatment were used. The test ran at 20ºC with 16:8 light/dark photoperiod. Soil moisture content was adjusted once a week by replenishing weight loss with the appropriate

amount of deionised water. The duration of the tests was two days (no food supply in this case) and three weeks. At the end of each exposure period the organisms were frozen in liquid nitrogen and stored at -80ºC until further analysis.

2.3.4. ChE activity

All the organisms in each replicate were homogenized in 1400 µl Buffer K-Phosphate 0.1M, pH 7.4. The tissue homogenate of each sample was centrifuged during 20 minutes at 10000 g (4ºC) to isolate the Post-Mitochondrial Supernatant (PMS). The ChE activity was determined as described above.

2.4. *In vitro* effects of copper and phenmedipham on ChE activity

The remaining PMS after the ChE characterization was used to determine the *in vitro* effects of Copper and Phenmedipham on ChE activity. This determination was done as described above except that Copper and Phenmedipham where added directly to the reaction solution. The concentrations of copper tested where: 0.003, 0.006, 0.012, 0.024, 0.048, 0.0975, 0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 µM. As for Phenmedipham, it was added as: 0.04875, 0.0975, 0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 g/l.

2.5. Statistical analysis

SIGMASTAT 3.5 (SPSS, 1997) software was used for statistical analysis. Data were tested for normality (Kolmogorov-Smirnov Test) and homoscedasticity (Bartlett's test). Results for the *in vivo* effects were analysed through a two-way ANOVA for differences between treatments and the Holm-Sidak test as used to discriminate statistical significant differences by performing multiple comparisons relatively to the control group. For the *in vitro* effects, one-way ANOVA was used to compare different treatments and the Dunnett test was used to discriminate statistical significant differences relatively to the control group. Also for the *in vitro* effects, the regression probit module of SPSS Systems 12.0 (SPSS, 2003) was used to calculate the inhibition concentration values (IC50). Data for substrate

affinity were analyzed fitting experimental curves using the Michaelis–Menten equation, in order to determine the kinetic parameters of ChE: maximal velocity (V_{max}), Michaelis–Menten constant (K_m) and their ratio (V_{max}/K_m) that indicates the catalytic efficiency of the enzyme, using SIGMAPLOT 10.0 (SPSS, 2006).

3. Results

3.1. ChE characterization

The highest rates of substrate hydrolysis were obtained with propionylthiocholine (PTCh) followed by acetylthiocholine (ATCh) and finally butyrylthiocholine (BTCh) (Figure 1). *E. albidus* ChE activity parameters K_m and V_{max} for each of the substrates used is presented in Table 2. The ratio K_m/V_{max}, which indicates the enzymatic catalytic efficiency, also clearly shows the enzyme preferences: PTCh>ATCh>BTCh (Table 2).

Table 2: Values of the Michaelis–Menten constant (K_m), maximal velocity (V_{max}) and the catalytic efficiency of *E. albidus* ChE (ratio K_m/V_{max}) for the three substrates tested.

	K_m mM	V_{max} nmol/min/mg protein	V_{max}/K_m µl/min/mig protein
Acetylthiocholine	0.16±0.019	15.32±0.48	96.48±8.58
Propionylthiocholine	0.17±0.0049	23.36±0.46	137.49±2.40
Butyrylthiocholine	0.24±0.074	19.70±2.07	85.22±16.62

The results obtained for the inhibitors in the presence of ATCh as substrate (Figure 2) showed that increasing concentrations of iso-OMPA did not alter ChE activity ($F=1.309$; $df=43$; $p=0.269$). As for Eserine and BW284C51, both strongly decreased ChE activity with increasing concentrations of the inhibitors, showing statistically significant differences relatively to the control from the first inhibitor concentration, 0.781 µM (Eserine: $F=84.805$; $df=39$; $p<0.001$; BW284C51: $F=171.967$; $df=43$; $p<0.001$). At the first inhibitor concentration, 0.781 µM, eserine and BW284C51 inhibit ChE activity, respectively, 55% and 38%. The last inhibitor concentration, 800 µM, inhibits ChE activity 20% and 15% for eserine and BW284C51 respectively.

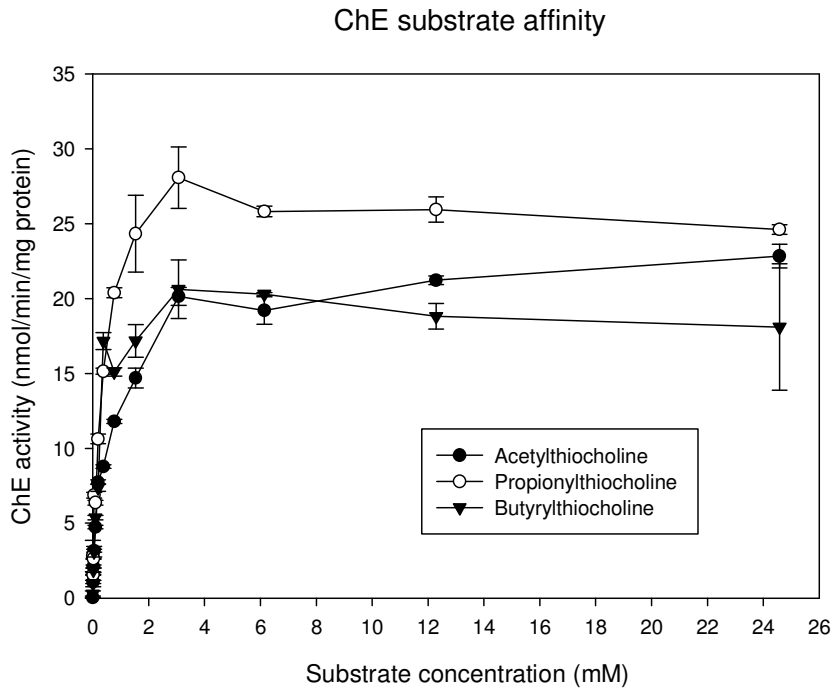

Figure 1: Results for ChE substrate preferences (ChE activity expressed as mean values ± standard error) for *E. albidus*.

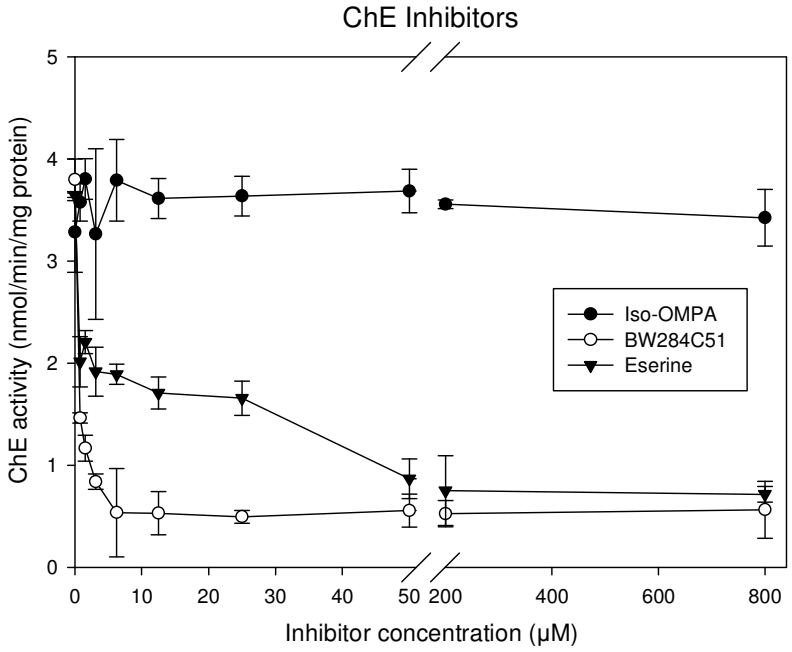

Figure 2: Effects of the ChE inhibitors iso-OMPA, BW284C51 and eserine on *E. albidus* ChE activity (expressed as mean values ± standard error) with acetylthiocholine as a substrate.

3.2. *In vivo* effects of soil properties, copper and betanal on ChE activity

Results for ChE activity in *E. albidus* exposed to LUFA 2.2 natural soil and to OECD standard artificial soil, during 2 days and 3 weeks show lower activities in the artificial soil for both time periods (Figure 3).

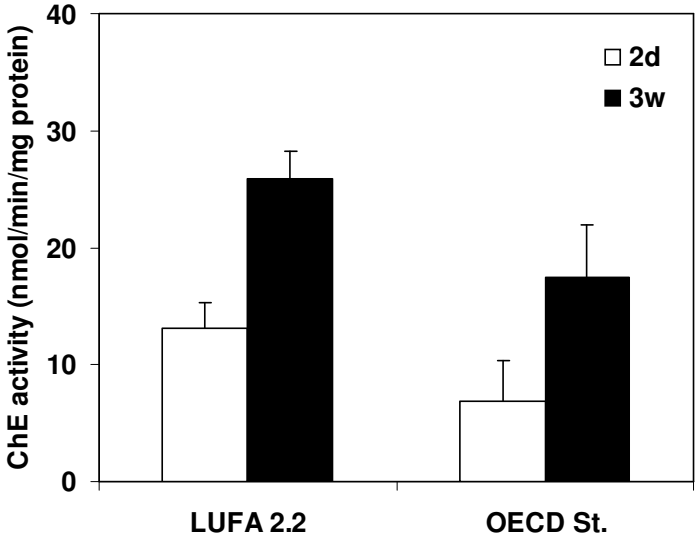

Figure 3: Results of ChE activity expressed as mean values (± standard error) for *E. albidus* exposed to LUFA 2.2 natural soil and OECD standard artificial soil (OECD St), for 2 days and 3 weeks. (* indicates statistically significant differences, Holm-Sydak $p<0.05$).

Results for ChE activity in *E. albidus* exposed to different soil properties after 2 days show higher activities in soils with lower organic matter and statistically higher activities ($p<0.05$) in soils with lower pH (Figure 4). After 3 weeks exposure, no statistically significant difference is observed, however, soils with higher clay content and with lower organic matter content show increased ChE activities (Figure 4).

Results for *E. albidus* exposed to copper and betanal show no statistically significant differences after 2 days exposure, although in soils spiked with copper ChE activities are visibly decreased compared to the control (Figure 5). After 3 weeks exposure, E*. albidus* ChE activities in soils spiked with betanal 32mg/Kg and Cu 320mg/Kg are statistically significantly decreased.

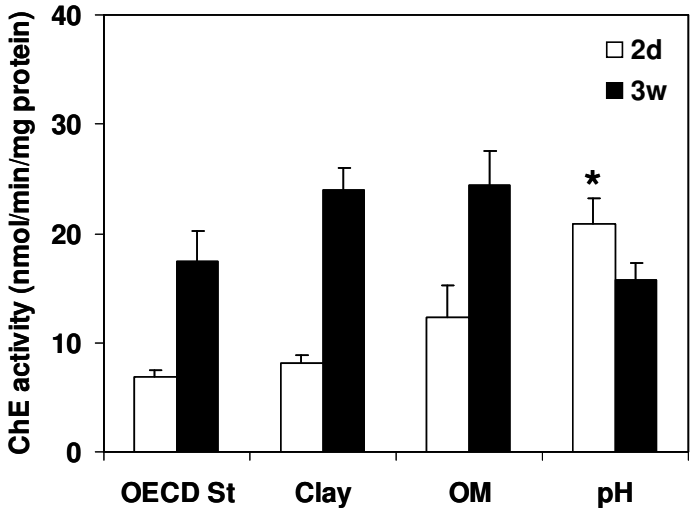

Figure 4: Results of ChE activity expressed as mean values (± standard error) for *E. albidus* exposed to OECD standard soil (OECD St), OECD soil with a high clay content (Clay), OECD soil with a low organic matter content (OM) and OECD soil with a low pH (pH), for 2 days and 3 weeks. (* indicates statistically significant differences, Holm-Sydak $p<0.05$).

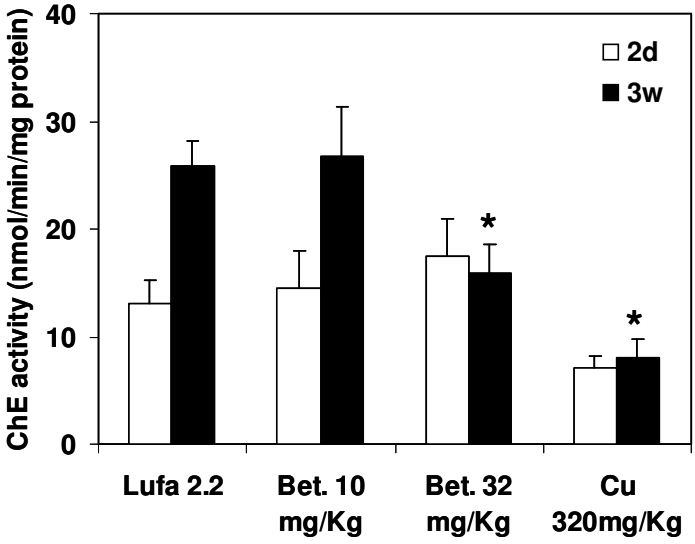

Figure 5: Results of ChE activity expressed as mean values (± standard error) for *E. albidus* exposed to LUFA 2.2 control soil and contaminated with Betanal (Bet.) and Copper (Cu) for 2 days and 3 weeks. (* indicates statistically significant differences, Holm-Sydak $p<0.05$).

3.3. *In vitro* effects of copper and betanal of ChE activity

Increasing concentrations of copper decreased ChE activity in *E. albidus* in vitro, with statistically significant differences compared to the control from 0.012µM of copper (F=63.362; df=71; p<0.001) (Figure 6). IC50% value determined for copper contamination in vitro was 1.08 µM (95% confidence limits: 0.93 – 5.57) and total inhibition of ChE activity occurs at 25 µM of copper (Figure 6).

Similarly, increasing amounts of betanal decreased ChE activity in *E. albidus* in vitro, with statistically significant differences compared to the control from 0.195 g/L of betanal (F=317.619; df=51; p<0.001) (Figure 7). IC50% value determined for copper contamination in vitro was 23.68 µM (95% confidence limits: 9.19 – 70.57), however, in this case, total inhibition of ChE was not observed for the concentrations tested (Figure 7).

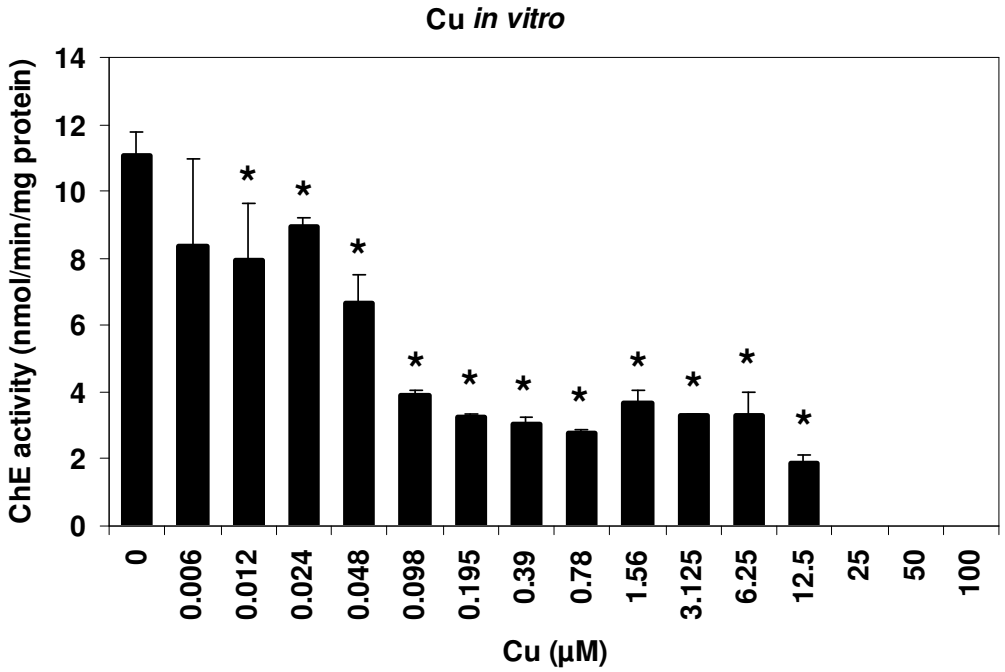

Figure 6: Results of ChE activity expressed as mean values (± standard error) for *E. albidus* exposed *in vitro* to copper. (* indicates statistically significant differences, Dunnett's p<0.05).

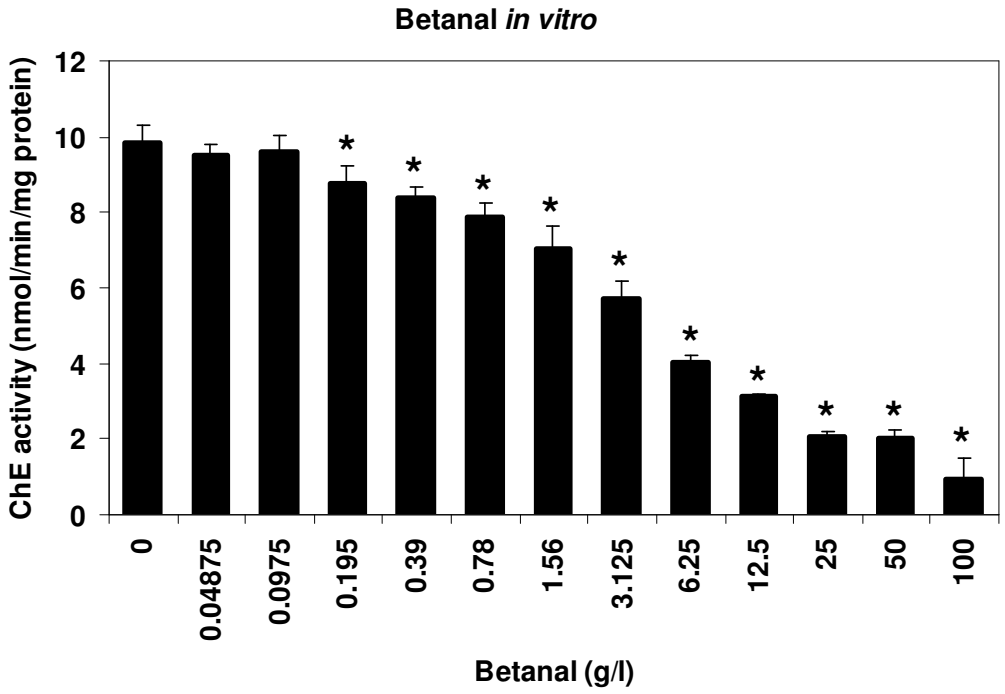

Figure 7: Results of ChE activity expressed as mean values (± standard error) for *E. albidus* exposed *in vitro* to Betanal. (* indicates statistically significant differences, Dunnett's $p<0.05$).

4. Discussion

ChE characterization

E. albidus possess only one ChE which displays mixed properties of vertebrate AChE and invertebrate ChE. AChE shows a high rate of hydrolysis when ACTh is used as a substrate, a relatively lower hydrolysis rate when PTCh is used and a very low activity with BTCh (Vellom et al., 1993). Although the results obtained for *E. albidus* ChE substrate preferences did not show a clear preference for ATCh in relation to BTCh (Figure 1), the inhibition assay (Figure 2) clearly showed that this ChE is highly sensitive to eserine sulphate, a potent inhibitor of ChE (Eto, 1974) and to BW284C51 the AChE selective inhibitor (Xu & Bull, 1994), however, it is not sensitive at all to Iso-OMPA, a strong inhibitor of BChE but not AChE (Barabona & Sánchez-Fortún, 1999).

Further studies should be performed in order to further characterize this ChE, including recovery studies from exposure to several classes of pesticides. The classification of ChE in invertebrates presents some difficulties since they are highly polymorphic enzymes encoded by a number of genes which varies among species and whose products may be characterized by different biochemical properties. Several ChE isoforms have been identified in invertebrates on the basis of substrate preference, for example, AChE and BChE in *Ostrea edulis* (Valbonesi et al., 2003), AChE and PChE in *Corbicula fluminea* (Mora et al., 1999), or by the use of specific blockers in the Antarctic scallop *Adamussium colbecki* (Corsi et al., 2004). In what regards earthworms, the phylogenetically closest organisms to enchytraeids, two types of ChEs are reported for *Eisenia. foetida*, that do not closely match the definitions of AChE and BChE, showing differences in their activity recovery after inhibition (Stenersen, 1979c, Mikalsen et al., 1982) and are addressed to as propionylcholinesterase and non-specific ChE (Sterensen, 1980; Ribera et al., 2001); and for *E. andrei* ChE activity, on the basis of substrate preference and the effects of specific inhibitors, appears to be mainly represented by AChE (Caselli et al., 2006).

Effects of soil properties, copper and betanal on ChE activity

LUFA 2.2 natural soil seems to be the most appropriate soil for *E. albidus,* since ChE activity is higher in this soil, for both exposure periods than in OECD artificial soil (Figure 3). This results are consistent with previous oxidative stress biomarkers studies in *E. albidus* were the biomarker responses analysed (TG, GST, Gpx, GR, CAT, SOD and LPO) were stable and not time-dependent in LUFA 2.2 soil and highly variable and time-dependent in OECD soil (Howcroft et al., 2008). Other studies with *E. albidus* and *E. crypticus* also showed that the substrates used for culturing these organisms influence oxidative stress biomarkers (Suteková et al., 2007). Current results are also in good agreement with previous reproduction and avoidance behaviour studies using *E. albidus* (Amorim et al., 2005a, b) where the reproductive output is higher in LUFA 2.2 than in OECD soil and that OECD soil is avoided when LUFA 2.2 soil is available.

ChE activity in *E. albidus* exposed to modified OECD soils showed no inhibition (Figure 4). However, OECD pH induced a higher activity than the OECD St. soil. Previous studies concerning avoidance and reproduction tests (Amorim et al., 2005 b, c, 2008 a) clearly showed that *E. albidus* prefer non-modified OECD standard soil than OECD soils with modifications of pH, clay and organic matter content. Current results show that ChE activity can not be used as a biomarker for the soil properties clay, organic matter and

In vivo contamination with Copper and betanal 32mg/Kg inhibited ChE activities after 3 weeks exposure and *in vitro* studies with these two chemicals showed that increasing concentrations/amounts decrease ChE activity (Figure 5). Previous results obtained by Amorim et al. (2005 c, 2008 b) showed that adult mortality LC_{50} for copper is higher than 320 mg/Kg, that the reproduction EC_{50} is 97 mg/Kg and that the AC_{50} for the avoidance test is 132.6mg/Kg, showing that this biomarker is a good sub-lethal stress marker that might be associated with effects at higher levels of biological organization. Results obtained for Phenmedipham contamination also seem to be in good agreement with decreased reproduction observed on *E. albidus*, EC_{50}= 29mg/Kg (Amorim et al., 2005 b). Since the results for the avoidance and mortality tests were AC_{50}=50.7 mg/kg LC_{50}=50 mg/kg (Amorim et al., 2005 a, 2008 b), it seems that ChE activity can also be used as a sub-lethal biomarker for this herbicide. Another interesting aspect is related to the results obtained for the avoidance test in Amorim et al. (2008b) in which the AC_{50} was 7mg/kg and, for concentrations higher than 10mg/kg, the organisms were not able to avoid the contaminated soils with higher concentrations. This fact could be explained by the decreased ChE activity obtained in the present work.

The result obtained for copper and betanal *in vivo* and *in vitro* are also consistent with *E. albidus* oxidative stress biomarkers determination (Howcroft et al., 2008), which showed significant alterations due to these chemicals (Figures 6 and 7). Furthermore, these results are in good agreement with the fact that heavy metals (Gill et al., 1990b; Reddy & Venugopal, 1993; Labrot et al., 1996; Guilhermino et al., 1998; Frasco et al., 2005) and several classes of pesticides are anti-ChE substances (Davies & Cook, 1993; Gill et al., 1990a; Guilhermino et al., 1998; Dembele et al., 1999) in several vertebrate and invertebrate species. Among these

species, studies of ChE activities in earthworms, including *Eisenia foetida*, *Aporrectodea calignosa*, *Lumbricus terrestris* and *Drawida willsi*, have also been shown to be inhibited by several classes of pesticides (Stenersen, 1979a, b; Drewes & Vining, 1984; Drewes et al., 1987; Booth et al., 2000; Panda & Sahu, 2004; Rodríguez-Castellanos & Sanchez-Hernandez, 2007), and have allowed the linkage with behaviour.

ChE activity has been enzymologically characterized in *Eisenia foetida* and *Eisenia andrei* (Stenersen, 1980; Caselli et al., 2006) and these earthworms ChE activity has been show to be drastically inhibited by organophosphorous (OP) and carbamate (CB) pesticides (Rao et al., 2003; Ribera at al., 2003), like in many other organisms, both in the laboratory and in the field (Fulton & Kev, 2001; Hill, 2003). The ChE inhibition in earthworms has been proven to be more sensitive than other biological endpoints commonly used during toxicity testing in earthworms. For example, ChE inhibition measured in juvenile *Aporrectodea caliginosa* was a more sensitive indicator of OP toxicity at low exposure levels than toxicity end points such as survival, growth, or reproduction (Booth & O'Halloran, 2001). Therefore, and due to current results, it is possible that ChE activity in *E. albidus* could be used as a more sensitive indicator of betanal and copper.

The results for the two exposure periods showed differences and the 3 week period seems to be the most appropriate for ChE studies in *E. albidus*, allowing a correct assessment of the influence of the stressors on the organisms. These results are consistent with previous oxidative stress biomarkers studies in *E. albidus* (Howcroft et al., 2008).

Further studies should include behavioural, growth, locomotory and feeding studies with *E. albidus* in order to assess the linkage of ChE activity with these commonly affected processes.

5. Conclusions

E. albidus possess one ChE that displays mixed properties of vertebrate AChE and invertebrate ChE.

ChE activities are not inhibited by the soil properties clay, organic matter and pH and therefore this enzymatic activity can not be used as a biomarker for these natural stressors.

Copper and betanal exposure inhibit ChE activities in *E. albidus* after 3 weeks exposure, showing that this biomarker can be used as can be used as an early-warning tool for these chemicals in biomonitoring studies.

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Chapter 4

Differential gene expression analysis in *Enchytraeus albidus* exposed to natural and chemical stressors: effect of different exposure periods

Differential gene expression analysis in *Enchytraeus albidus* exposed to natural and chemical stressors: effect of different exposure periods

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Abstract

The soil oligochaete *Enchytraeus albidus* is a test organisms used in biological testing for soil Environmental Risk Assessment (ERA), with a standardized protocol. Although effects are known at acute and chronic level, with survival, reproduction and avoidance endpoints, very little is known at the sub-cellular and molecular levels. In this study, the effects of soil properties (clay, organic matter and pH) and of the chemicals copper and phenmedipham were studied on gene expression in *E. albidus* during exposure periods of 2 days (previous results from Amorim et al., 2008 submitted), 4 days and 3 weeks, using a DNA microarray for this test species, based on a normalised cDNA library (Amorim et al., 2008 submitted). The objective of this study was to obtain stress markers, which can be used to discriminate stress of unfavourable soil properties and toxic stress, as well as assessing the effect of the time of exposure in gene expression and determining the best exposure period for gene expression analysis. In total, hybridization revealed 117 differentially expressed genes. A different pattern of differentially expressed genes was observed for the conditions studied: abiotic factors induce the differential expression of a higher number of genes. Different exposure periods induced differences in gene expression, depending on the type of stress: for chemicals shorter exposure periods induce a higher number of differentially expressed genes, which diminish with increasing exposure periods; for abiotic factors shorter exposure periods alter the expression of a lower number of genes compared to longer exposure periods.

Keywords: Enchytraeids; microarray; differential gene expression; soil properties; phenmedipham; copper.

1. Introduction

The family Enchytraeidae belongs to the saprophagous mesofauna of the litter layer and the upper mineral soil contributing to vital processes of this environmental compartment, directly by improving the pore structure of the soil and by being indirectly involved in regulating the degradation of organic matter (Amorim *et al*., 2005 a). *Enchytraeus albidus* is the best-known and one of the largest species of the genus *Enchytraeus*, having been used primarily in single chemical assays with OECD artificial soil or LUFA 2.2 natural standard soil, as well as being successfully used for the assessment of soil quality (Jänsch *et al.* 2005). Enchytraeis have established standardized procedures (ISO, 2003; OECD, 2003 and ASTM, 2004), for biological effect assessment and extensive studies on the effect of different chemicals, including copper and phenmedipham, and soil proprieties on *E. albidus* have been performed by Amorim *et al.* (2005a, b, c and 2008 a, b) using mortality, reproduction and avoidance behaviour endpoints. Although these types of effect assessments are consistent and important to predict threshold values for policy makers and to screen polluted soils for toxicity, they are time consuming and may underestimate effects occurring at the sub-cellular and molecular levels. Recently oxidative stress biomarkers and cholinesterase activity have been performed in order to further unravel the effects of soil properties, copper and phenmedipham on *E. albidus* (Howcroft et al., 2008). However, the exact mechanistic toxicity of these stressors on these organisms has still to be determined.

Copper and phenmedipham were chosen for this study because they are representative of two different groups of chemical stressors with different properties, present commonly in soils, metals and herbicides and due to previous knowledge of effects at population level (2005 a, b, c, 2008). Known effects of these chemicals on *E. albidus*, include avoidance behaviour, survival and reproduction (copper chloride: LC_{50}=320mg/kg, AC_{50}=132.6mg/kg, EC_{50}=97mg/kg; Phenmedipham: AC_{50}=50.7 mg/kg, LC_{50}=50 mg/kg and EC_{50}=29.4 mg/kg).

The rapid progress in the development of molecular biological techniques has provided tools that may assist our understanding of how chemicals can affect ecosystems. Through gene expression analysis after toxicant exposure, molecular

mechanisms underlying toxic responses to environmental contaminants can be unravelled, allowing the assessment of population and community level effects, rather than only effects on individuals. This emerging field of ecotoxicogenomics could be very helpful to identify mechanisms of action of chemicals in standard terrestrial invertebrate test organisms whose genomes are still poorly characterized, such as *E. albidus*, similarly to what has already been done with the aquatic crustacean *Daphnia magna*, as well as with the earthworms *Eisenia fetida* (Pirooznia et al., 2007) and *Lumbricus rebellus* (Bundy et al., 2008).

Differential gene expression analysis can be assessed through various strategies, one of which is the use of DNA arrays (Moens *et al.*, 2003; Larkin *et al.*, 2003). One of the types of DNA arrays consists of small glass slides (microarrays) embebed with ordered rows of small quantities of oligonucleotides or cDNAs, which allow the researcher to directly measure changes in the transcriptional regulation of many thousands of genes simultaneously in a single analysis (Pennie, 2002). By investigating the effects at the mRNA level (using microarrays) we can derive biomarkers that can be used to discriminate stress induced by unfavourable soil properties from stress responses caused by toxic compounds. Amorim et al. (2008 submitted), had previously designed a custom-made DNA microarray for *E. albidus*, based on a normalised cDNA library, and the results from this study revealed that after 2 days exposure to different soil properties and chemicals (copper and betanal) gene expression was altered.

In the present study, organisms were exposed to copper, phenmedipham and different soil types during two exposure periods, 4 days and 3 weeks, using the existing DNA microarray for *Enchytraeus albidus*. Results were also compared to results of the 2 day exposure (Amorim et al., 2008 submitted). The main objectives of this study were to evaluate if the different exposure periods lead to differences at the transcription level, determine the ideal exposure period and obtain gene biomarkers for the exposures.

2. Material and Methods

2.1. Test organism

Laboratory cultures of the test species *Enchytraeus albidus* (Henle, 1837) were maintained in moist soil (50% OECD soil, 50% natural garden soil), at 18ºC with a photoperiod of 16:8h (light:dark), and fed once a week with finely ground and autoclaved rolled oats (Cimarrom, Portugal). Details of the culturing process are given in Römbke and Möser (2002).

2.2. Test soils

Two standard soils were used: natural LUFA 2.2 soil, from Speyer, Germany (Lokke & Van Gestel, 1998) and OECD artificial soil (OECD, 1984). Different OECD artificial soils were produced, manipulating its composition in terms of percentage of clay, sand, peat content and pH value. The main characteristics of the test soils used are presented in table 1.

Table 1: Main characteristics of the test soils, showing approximate values for: grain size distribution (sand, clay, silt), organic matter content (OM), pH and Water Holding Capacity (WHC).

Soil Type	Sand (%)	Clay (%)	Silt (%)	OM (%)	pH ($CaCl_2$ 0.01M)	WHC
LUFA 2.2.	79	13	8	2.36	5.6	48
OECD St.	70	20	-	10	6.3	80
OECD-clay	20	70	-	10	6.4	107.5
OECD-pH	70	20	-	10	5	59
OECD-OM	72.5	22.5	-	5	6.8	47.5

2.3. Test chemicals

Phenmedipham, an herbicide, was applied as the formulation Betosyp formerly known as Betanal (STÄHLER AGROCHEMIE, 157 g/L a.i.) to LUFA 2.2 soil in the following nominal concentrations: 10 and 32 mg a.i./Kg DW (Dry Weight). Copper chloride (di)hydrated ($CuCl_2 \cdot 2H_2O$; purity, 99%; molecular weight 170.48 g/mol;

Merck, Darmstadt, Germany), was added as an aqueous solution to the soil in order to give a final concentration of 320 mg/kg DW. The contamination of all test substrates was done by mixing the aqueous solution of the test chemical into the pre-moistened LUFA 2.2 soil. After homogenous mixing, sub-samples of soil were introduced into the individual test vessels. In the case of the metal, the soil was allowed to equilibrate three days before test start as recommended by McLaughlin (2002).

Concentrations were selected based on previous results (Amorim et al. 2005 a, b, c, 2008 a, b), being the selection based on selecting a chronic (reproduction) effect concentration, within the EC_{50} range.

2.4. Test procedure

Fifteen adult worms with well-developed clitellum were selected and introduced in a glass vessel (covered afterwards with a parafilm layer in which a few holes for airing were made), each containing 25 g moist soil (40–60% of the maximum WHC) plus food supply (50 mg of finely ground and autoclaved rolled oats, being half of the amount supplied every week). 8 replicates per control and 4 replicates per treatment were used. The test ran at 20ºC with 16:8 light/dark photoperiod. Soil moisture content was adjusted once a week by replenishing weight loss with the appropriate amount of deionised water. The duration of the tests was 4 days (no food supply in this case) and 3 weeks. At the end of each exposure period the organisms were placed in criotubes (with RNA later), frozen in liquid nitrogen and stored at -80ºC until further analysis.

2.5. Differential gene expression assessment

2.5.1. Spotting of cDNA microarrays

cDNA-inserts of the clones were loaded into 384-well plates (Genetix, UK) in 50% dimethylsulfoxide at 75 ng/L and a Agilent platform was used to spot the cDNA library (Amorim et al., 2008 submitted) in triplicate onto aminosilane-coated glass slides (Generoma microarray slides; Asper Biotech, Estonia), together with a set of artificial control genes containing calibration controls, ratio controls and negative

control genes (Lucidea Universal Scorecard, Amersham Biosciences, UK) that were spotted over the arrays in 15 replicates. These spiked-in labelled controls at known concentrations provide information on cDNA labelling efficiency, sensitivity and intra-array variability of replicates. Negative controls (cDNAs with no provided mRNA spike) were also included to assess non-specific hybridization and quality of blocking at the prehybridization step. After rehydration and drying, the slides were cross-linked by UV-radiation at 300 mJ (UV Stratalinker 2400; Stratagene, USA).

2.5.2 RNA extraction, cDNA synthesis, fluorescent labelling and microarray hybridization

Total RNA from *E. albidus* exposed for 4 days and 3 weeks to the different exposure conditions was isolated, by application of the Trizol extraction method (Invitrogen, Belgium) and a DNase treatment was performed (Fermentas, Germany). The RNA concentration was determined by spectrophotometry (app 1000 ng/µl) and the quality was checked by a denaturing formaldehyde–agarose gel.

3 replicates of DNase treated RNA from each exposure condition and a pool of the 3 control replicates were used to synthesise cDNA. Seven micrograms of the total RNA, spiked with the mRNA mix from the Lucidea Universal Scorecard (Amersham Biosciences), was converted into single-stranded cDNA with incorporated aminoallyl-dUTPs (Sigma, USA) through a random primer (Invitrogen SA, Belgium) extension with superscript II reverse transcriptase (Invitrogen). After purification with the QIAquick PCR purification kit (Qiagen Inc., USA), the aminoallyl-labeled cDNA was covalently coupled with Cy Dye esters (Amersham Biosciences). Aminoallyl cDNA from exposed and control enchytraeids were coupled, respectively, with Cy5 and Cy3 (or vice versa in dye-swap experiments). The uncoupled Cy3/Cy5 were removed by the QIAquick PCR purification kit (Qiagen) and labelling efficiency was determined by spectrophotometry. Each of the three biological replicates of the exposed enchytraeids was hybridized on a separate array against one labelled control pool for each exposure period.

Vacuum-dried probes containing 150 pmol of each incorporated dye were resolved in hybridization solution (50% formamide, 5% SSC, 0.1% SDS, 0.1 mg/mL BSA, 0.1 mg/mL salmon sperm) and incubated at 95ºC for 5 min, mixed and applied on the array slides.

Prior to the hybridization, arrays slides were incubated in a coupling jar with prehybridization solution (50% formamide, 5% SSC, 0.1% SDS, 0.1 mg/mL BSA) at 42ºC for 60 min. The arrays were then washed with deionized water (twice) and isopropanol and immediately dried with compressed N_2. After the application of the probes, the cover-slipped slides (Menzel Glaser, Germany) were placed in a hybridization chamber (Genetix) and hybridized at 42ºC overnight. Slides were then washed with the following wash buffers: 2% SSC and 0.1% SDS (at 42ºC for 1 and 5 min), 0.1% SSC and 0.1% SDS (room temperature twice for 10 min), 0.1% SSC (room temperature for 15 s, twice for 2 min and 1 min) and 0.01% SSC (room temperature for 15 s). Finally slides were rinsed twice with deionized water, once with isopropanol and dried with compressed N_2.

2.5.3. Scanning and bioinformatics analysis

Microarrays were scanned at 532 and 635 nm using the Agilent DNA Microarray Scanner G2565B.

The images were analyzed using QuantArray Software for spot identification, quality assessment and quantification of the fluorescent signal intensities. Low intensity spots and saturated signals were filtered out. The data obtained was then analysed using the software BRB-Array tools version 3.7.0 to calculate the fluorescent signal intensity for each DNA spot (average of intensity of each pixel present within the spot) with background subtraction. The $\log_2$ ratio (Cy5/Cy3) was calculated for each spot and normalized using Locally Weighed Scatterplot Smoothing (Lowess) (Yang et al., 2002). All analyses were based on triplicate experiments (biological replicates) and triplicate spots per gene on each array, resulting in nine measurements per gene per condition. As means of control, 3 dye swaps were performed, as well as 3 self-self experiments (RNA from a single pool was labelled with both dyes and hybridized to the same arrays).

In order to analyse the differences between the exposure periods and the conditions studied, 4 days and 3 week results were compared with the 2 days results (Amorim et al., 2008 submitted) by determining the median of the 3 biological replicates for each condition and differential gene expression was determined using the cut-off values of ±0.8 (Amorim et al., 2008 submitted).

A heat map of the fold-induction results was produced for all the exposure periods using Multi Experiment Viewer (MEV) version 4.1. The hierarchical average linkage clustering of the genes and samples was done using the Pearson uncentered distance metric.

The cDNA fragments of the microarray had previously been sequenced and identified based on their homology to sequences from the National Center for Biotechnology Information (NCBI) database as determined by the Basic Local Alignment Search Tool (BLAST). Both BLASTN and BLASTX were performed to identify the isolated clones by comparing with DNA and proteins databases respectively (Amorim et al., 2008 submitted). All genes were annotated using gene ontology (http://www.geneontology.org) and classified by function.

3. Results

All arrays performed in the analysis fulfilled the required quality parameters (e.g. no nonspecific hybridizations). Additionally, to correct for possible dye-biases, dye-swap experiments were carried out.

Figure 1 shows the heat map for the differentially expressed genes in the microarrays produced for all the exposure periods (2 days, 4 days and 3 weeks) and stress conditions, as well as the detailed sample cluster for all the genes analysed. The clustering of all the samples analysed separated within the 2 and 4 days exposure period the two types of stress, abiotic and chemical. However for the 3 week exposure this distinction was not observed.

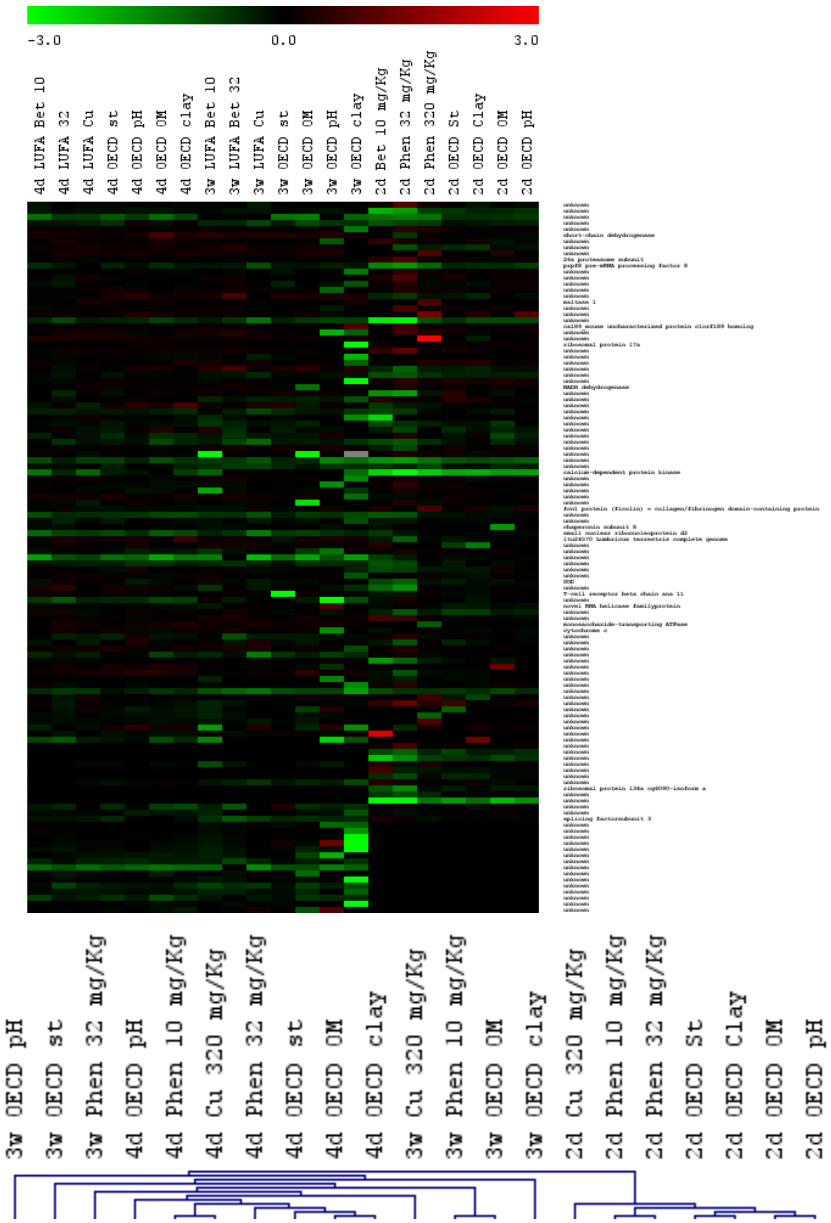

Figure 1: Heat map for the differentially expressed genes for all the exposure periods (2 days, 4days and 3 weeks) and stress conditions, and detail of the sample clustering for all the genes in the microarray.

Of the 480 cDNAs spotted on the array, a total of 117 genes were found to be differentially expressed by the different stress conditions both in the two exposure periods determined in this study (4 days and 3 weeks) and in the 2 days exposure (data form Amorim et al., 2008), as determined by the significance thresholds for up and down-regulated genes (±0.8-fold, Amorim et al., 2008).

The percentages of differentially expressed genes are presented per functional category in figure 2 A.

Differential gene expression was also analysed for each time period and is presented in figure 2 (B, C and D), showing the differences in terms of the number of differentially expressed genes per category, for each time period.

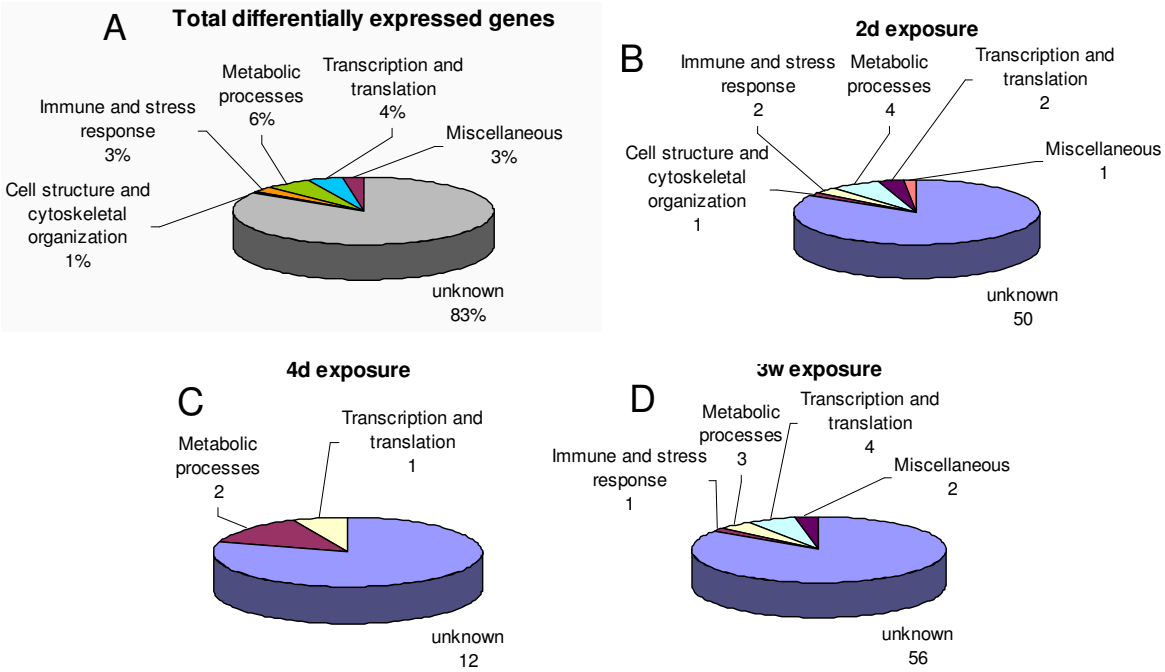

Figure 2: Number and distribution of the total differentially expressed genes per functional category (A) and also for 2 days (B) 4 days (C) and 3 weeks (D) exposure.

The number of differentially expressed genes was not the same for chemical and abiotic stress, and also varied within each according to the duration of the exposure. Abiotic factors affected genes involved in immune and stress response, metabolic processes, transcription and translation (table2). The chemical substances studied altered the expression of genes involved in cell structure and cytoskeletal organization, immune and stress response, metabolic processes, transcription and translation (table 3).

Results for the abiotic stress show that higher clay content and lower organic matter content as well as lower pH tend to induce higher differential gene expression with increasing exposure periods (table 2; figure 4). On the other hand, chemical stress results show that there is tendency to a decrease in differentially expressed genes with increasing exposure periods (table 3; figure 5).

Table 2: Number of differentially expressed genes induced by exposure to the OECD soils (artificial standard and modified OCDE soils) in *E. albidus*.

	OECD total	OECD 2d	OECD 4d	OECD 3w
unknown	71	13	11	46
Cell structure and cytoskeletal organization	0	0	0	0
Immune and stress response	2	1	0	1
Metabolic processes	4	1	1	3
Transcription and translation	3	0	1	2
Miscellaneous	0	0	0	1
Total	70	15	13	53

Table 3: Number of differentially expressed genes induced by exposure to copper and phenmedipham in *E. albidus*.

	Chemicals total	Chemicals 2d	Chemicals 4d	Chemicals 3w
unknown	55	43	8	19
Cell structure and cytoskeletal organization	1	1	0	0
Immune and stress response	1	1	0	0
Metabolic processes	4	4	1	1
Transcription and translation	3	2	1	2
Miscellaneous	2	1	0	1
Total	66	52	10	23

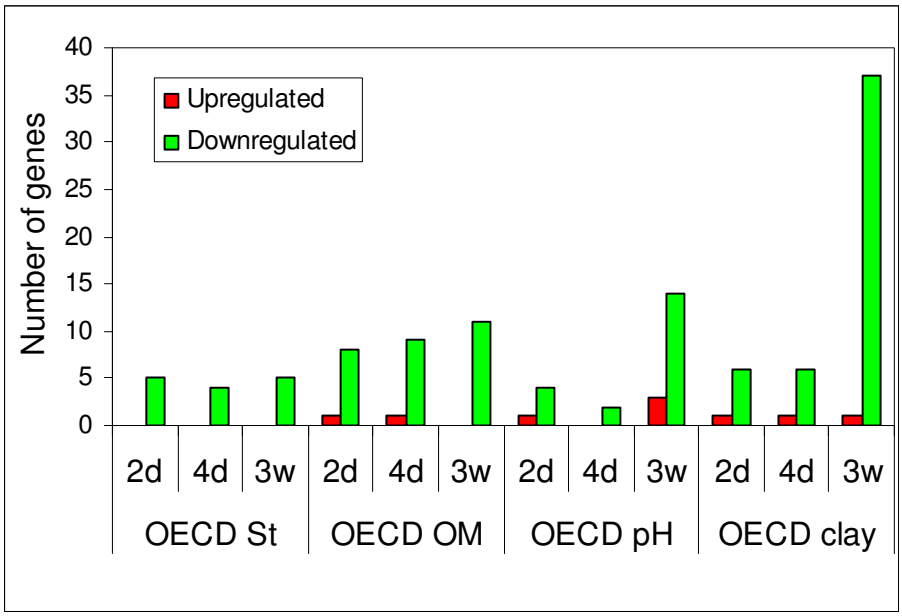

Figure 3: Number of up and down-regulated genes for the OECD standard soil and OECD soil variations, for each time period.

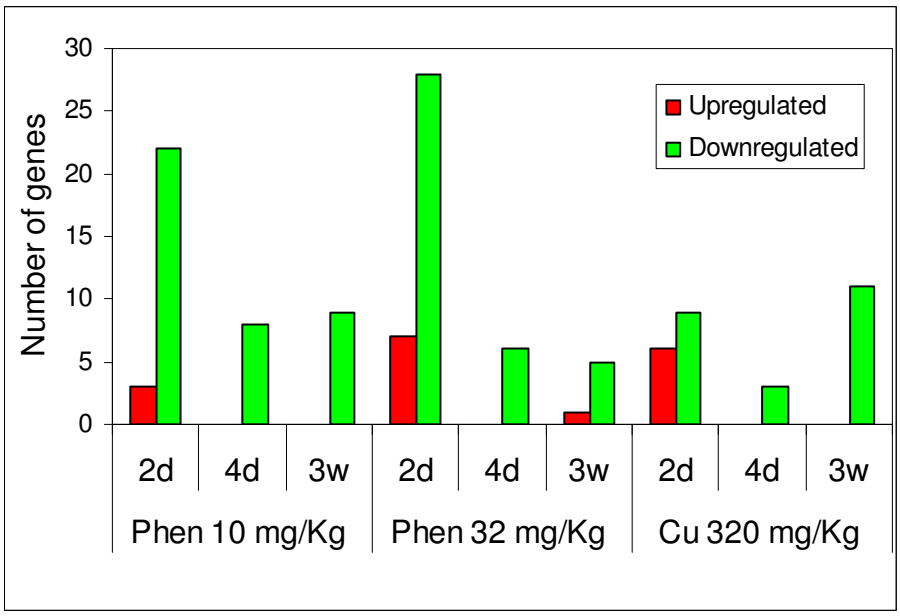

Figure 4: Number of up and down-regulated genes for the chemical substances spiked in LUFA 2.2. soil, for each time period.

4. Discussion

Abiotic stress induced in total, for all the exposure periods, the differential expression of a higher number of genes than chemical stress (tables 2 and 3).

However, analysing the results for each exposure period (tables 2 and 3; figures 4 and 5), while abiotic stress tended to increase differential gene expression with increasing exposure period, chemical stress tended to decrease the number of differentially expressed genes with increasing exposure periods. This could be due to the fact that the organisms are more induced to respond to chemical stress in a stronger way, and adapt to the stress as the exposure period increases. This is also visible in the fact that after only 2 days of exposure the number of differentially expressed genes is much higher for chemical stress than for abiotic stress (tables 2 and 3).

Phenmedipham exposure for 2 days induced a higher number of differentially expressed genes in *E. albidus* for both concentrations, which diminished progressively with the increase in the exposure period (figure 5). As for copper, after 2 days of exposure a high number of down-regulated and up-regulated genes were observed. At 4d exposure the number of differential gene expression decreased but after 3 weeks the number of down-regulated genes increased and it was even higher than in the 2 days exposure.(figure 5). These are results are consistent with oxidative stress biomarkers determinations (Howcroft et al., 2008), which showed that copper affected these endpoints more significantly than phenmedipham. In the same study the assessment of lipid peroxidation after 3 weeks exposure both to copper and phenmedipham showed that *E. albidus* was facing oxidative stress damage, which may be related with gene expression regulation.

All the abiotic factors studied is this research work affected gene expression, particularly low pH and high clay content. These results are consistent with previous studies concerning avoidance and reproduction tests (Amorim et al. 2008a) that clearly showed that *E. albidus* prefer non-modified OECD standard soil than OECD soils with modifications of pH, clay and organic matter content. Oxidative stress biomarkers analysis (Howcroft et al., 2008) also showed that these abiotic factors decrease antioxidant capacity although there was an absence of oxidative stress assessed as lipid peroxidation.

Both types of stress factors affected genes involved in important biological functions: abiotic stress affected genes involved in immune and stress response, metabolic processes, transcription and translation (table 2); chemical stress altered the expression of genes involved in cell structure and cytoskeletal organization, immune and stress response, metabolic processes, transcription and translation (table 3). These functions have implications at higher ecologically relevant levels, as is seen given the possible link with population and behaviour endpoints.

Further analysis is still ongoing and results are still expected from RT-PCR in order to quantitatively assess gene expression differences for some of the genes. If the RT-PCR results would confirm the gene expression pattern of the microarray analysis then these genes can possibly be used as early warning signal for chemical stress since the results obtained by Amorim et al. (2005a,b,c; 2008b) show that copper and phenmedipham at the concentrations used diminish reproduction and affect avoidance behaviour. Therefore this discussion is still preliminary.

5. Conclusions

The abiotic factors studied (low pH, high clay and low organic matter content) affected genes involved in immune and stress response, metabolic processes, transcription and translation, and chemicals studied (copper and phenmedipham) altered the expression of genes involved in above functions and additionally genes involved in cell structure and cytoskeletal organization.

In total, for all exposure periods, chemical stress lead to the differential expression of a lower number of genes compared to abiotic factors However, genes affected by chemical stress are involved in a wider range of biological functions than the ones affected by abiotic factors, suggesting a more widespread effect.

The time period affects gene expression.

This study shows the association of the effects on gene expression with oxidative stress effects and with the alterations on higher levels of biological organization (survival, avoidance and reproduction endpoints) and may lead to the use of microarray analysis for biomonitoring studies with *E. albidus*. With the sequencing

of *E. albidus*' genome, it may be possible in the future to understand the underlying modes of action and biochemical pathways affected by different stress and pinpoint specific gene markers.

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Chapter 5

General Discussion and Conclusions

General discussion and conclusions

The work presented in this Masters thesis shows the result of research work into the use of oxidative stress, neuro-muscular and gene expression biomarkers for *Enchytraeus albidus* exposed to different abiotic factors and two types of chemicals, an herbicide and a metal. These results clearly showed that different types of stresses affect this organism at the sub-cellular and molecular levels in different ways and that these endpoints can be used in stress assessment in *E. albidus*.

The battery of biomarkers analysed in this research work was quite broad since this constituted the first study of this type in *E. albidus*. It therefore aimed at assessing their usage and applicability. For the chemical stress, induced by the heavy metal copper and the herbicide phenmedipham, the oxidative stress biomarkers catalase, glutathione peroxidase and glutathione-S-transferase appear to be consistent and good biomarkers for chemical stress assessment along with lipid peroxidation as an indicator of oxidative stress status, as well as the neuromuscular parameter determined, cholinesterase activity. The remaining biomarkers analysed show more variable responses and therefore their usage is not so strongly recommended in comparison with the previous ones, based on the results obtained.

As for the abiotic factors, oxidative stress biomarkers analysis points to the usage of glutathione peroxidase as the more robust biomarker for all the factors, and additionally glutathione-S-transferase for low organic matter content and pH value. The soil composition percentages of clay and organic matter as well as the pH value used in this research study did not cause oxidative stress assessed as lipid peroxidation. Cholinesterase activity was not influenced by the abiotic factors and therefore this neuromuscular parameter is not the most indicated biomarker for such abiotic stress assessment. The influence of these abiotic factors should also be studied in natural soil types since the results here presented are compared with an artificial soil which shows lipid peroxidation and significant alterations of oxidative stress enzymes activities and molecule levels when compared with a

natural soil and therefore may be inducing a bias in the statistical analysis and conclusions.

At the gene expression level, in general, the biological functions affected were cell structure and cytoskeletal organization, immune and stress response, metabolic processes and transcription and translation. These constitute key functions that can lead to individual and population level effects. In total, for all exposure periods, chemical stress lead to the differential expression of a lower number of genes compared to abiotic factors, however, genes affected by chemical stress are involved in a wider range of biological functions than the ones affected by abiotic factors, suggesting a more widespread effect.
However, due to the fact that *E. albidus*' genome has not been sequenced, results are constrained by the homology analysis of the DNA fragments in other organisms. Therefore, although the microarray showed very interesting results in terms of number of differentially expressed genes, in the future, studies on gene expression in *E. albidus* could be even more detailed if we could pinpoint more specifically which genes are being differentially expressed and hence determine which processes are being affected more accurately.

The use of natural soil types for ecotoxicological testing in *E. albidus* is strongly recommend due to alterations in biomarkers activities and levels as well as in gene expression. Also, linking the results from the biomarkers and gene expression analysis it becomes obvious that the exposure period is very important in these determinations. Of the exposure periods analysed, the 3 weeks period seems to be the more consistent and reliable since on the one hand it allows time for the organisms to reach a sort of equilibrium (past the initial response) and on the other hand allows the assessment of effects that are manifested only after some time of exposure. However, this period is still quite long and therefore further intermediate exposure periods should be studied.

This research work clearly points to the importance of assessing oxidative and neuromuscular biomarkers, as well as gene expression analysis together as

traditional acute and chronic endpoints in ecotoxicology (growth, reproduction, survival, avoidance behaviour). This allows relating stress effects at the population, sub-cellular and molecular levels for the further establishment of reliable faster-screening endpoints of stress effects in *E. albidus*. Further work should be done with more metals and herbicides, to determine if the endpoints here suggested can be applied to these vast groups of chemical substances. As for the abiotic factors studied, future work could include the analysis of their joint effect with chemical contamination as well as the study of different natural soils.

This work could constitute the first step into to the future of soil ecotoxicology using *E. albidus* leading to a possible fast and reliable way to determine soil contamination and to assess stress effects using early-warning markers.